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(54) Title: ASSAYS FOR GROWTH HORMONE SECRETAGOGUE RECEPTORS

(57) Abstract

An assay for the detection of growth hormone secretagogue receptors and growth hormone secretagogue related receptors is described. As these receptors are a member of the G protein coupled receptors, a subunit of the G protein must be present in order for expression to be detected. A similar assay is described where the presence of growth hormone secretagogues are detected.

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TITLE OF THE INVENTION

ASSAYS FOR GROWTH HORMONE SECRETAGOGUE RECEPTORS

FIELD OF THE INVENTION

This invention relates to an assay which involves identification of cell membrane receptors, specifically growth hormone secretagogoue receptors (GHSRs). By varying the protocol, receptor ligands can be identified, or the presence of a GHSR can be identified.

BACKGROUND OF THE INVENTION

Growth hormone (GH) is an anabolic hormone capable of promoting linear growth, weight gain and whole body nitrogen retention. Classically, GH is thought to be released primarily from the somatotroph cells of the anterior pituitary under the coordinate regulation of two hypothalamic hormones, growth hormone releasing factor (GHRF or GRF) and somatostatin. Both GHRF stimulation and somatostatin inhibition of the release of GH occurs by the specific engagement of receptors on the cell membrane of the somatotroph.

Recent evidence has been mounting which suggests that GH release is also stimulated by a group of short peptides termed the growth hormone releasing peptides (GHRP; GHRP-6, GHRP-2 [hexarelin]) These peptides are described, for example, in U.S. Patent No. 4,411,890, PCT Patent Pub. No. WO 89/07110, PCT Patent Pub. No. WO 89/07111, PCT Patent Pub. No. WO 93/04081, and *J. Endocrinol Invest.*, *15*(Suppl 4), 45 (1992). These peptides function by selectively bind to a distinct somatotroph cell membrane receptor, the growth hormone secretagogue receptor (GHSR). A medicinal chemical approach has resulted in the design of several classes of orally-active, low molecular weight, non-peptidyl compounds which bind specifically to this receptor and result in the pulsatile release of GH. Such compounds possessing growth hormone secretagogue activity are disclosed, for example, in the following: U.S. Patent No. 3,239,345; U.S. Patent No. 4,036,979; U.S. Patent No. 4,411,890; U.S. Patent No. 5,206,235; U.S. Patent No. 5,283,241; U.S. Patent No. 5,284,841; U.S.

Patent No. 5,310,737; U.S. Patent No. 5,317,017; U.S. Patent No. 5,374,721; U.S. Patent No. 5,430,144; U.S. Patent No. 5,434,261; U.S. Patent No. 5,438,136; U.S. Patent No. 5,494,919; U.S. Patent No. 5,494,920; U.S. Patent No. 5,492,916; EPO Patent Pub. No. 0,144,230; EPO Patent Pub. No. 0,513,974; PCT Patent Pub. No. WO 94/07486; PCT Patent Pub. No. WO 94/08583; PCT Patent Pub. No. WO 94/11012; PCT Patent Pub. No. WO 94/13696; PCT Patent Pub. No. WO 94/19367; PCT Patent Pub. No. WO 95/03289; PCT Patent Pub. No. WO 95/03290; PCT Patent Pub. No. WO 95/09633; PCT Patent Pub. No. WO 95/11029; PCT Patent Pub. No. WO 95/12598; PCT Patent Pub. No. WO 95/13069; PCT Patent Pub. No. WO 95/14666; PCT Patent Pub. No. WO 95/16675; PCT Patent Pub. No. WO 95/16692; PCT Patent Pub. No. WO 95/17422; PCT Patent Pub. No. WO 95/17423; PCT Patent Pub. No. WO 95/34311; PCT Patent Pub. No. WO 96/02530; *Science, 260*, 1640-1643 (June 11, 1993); *Ann. Rep. Med. Chem.*, *28*, 177-186 (1993); *Bioorg. Med. Chem. Ltrs.*, *4*(22), 2709-2714 (1994); and *Proc. Natl. Acad. Sci. USA 92*, 7001-7005 (July 1995).

The use of such orally-active agents which stimulate the pulsatile release of GH would be a significant advance in the treatment of growth hormone deficiency in children and adults as well as provide substantial benefit under circumstances where the anabolic effects of GH might be exploited clinically (e.g. post-hip fracture rehabilitation, the frail elderly and in post-operative recovery patients).

Cell membrane receptors which are of low abundance on the cells can be difficult to isolate, clone and characterize. In the past, assays to identify a receptor in a mammalian cell or frog oocyte generally have depended on either: 1) directly detecting a receptor-ligand interaction, such as by binding of a radiolabeled ligand; or 2) indirectly detecting receptor-ligand binding by detecting either an intracellular event (such as calcium mobilization, or the identification of, for instance a calcium activated current) or an extracellular event (such as hormone secretion), that is the consequence of the ligand

binding to its receptor. Most cloned receptors, which have been isolated using a functional expression assay have relied on immortalized cell lines or tumor derived tissues which are enriched for the receptor of interest.

There are numerous receptors which cannot be readily identified using these types of assays, due to: 1) a paucity of biochemical information about the protein; 2) the low abundance of receptors present on the cell; and/or 3) the lack of a cell line or tumor material expressing the receptor. It would be desirable to develop an assay which can be used to identify and characterize cell receptors not amenable to study by conventional means.

DETAILED DESCRIPTION OF THE INVENTION

This invention relates to an assay method to determine the presence of a nucleic acid which encodes a G protein-linked cell membrane receptor comprising: a) introducing at least one nucleic acid suspected of encoding a G protein cell membrane receptor into a cell;

b) introducing a G-protein subunit into the cell;

c) introducing a detector molecule or a nucleic acid encoding a detector molecule into the cell, wherein the detector molecule responds directly or indirectly to a G-protein receptor-ligand binding event;

d) contacting the cell with a receptor ligand; and

e) determining whether the oligonucleotide encoded a receptor by monitoring the detector molecule.

In one preferred embodiment the cell does not naturally express the receptor on its cell membrane. In other preferred embodiments of the assay, the receptor is a member of the growth hormone secretagogue family of receptors, such as a growth hormone secretagogue receptor (GHSR) or a growth hormone secretagogue related receptor (GHSRR). Thus, another aspect of this invention is an assay method to determine the presence of a nucleic acid which encodes

a member of the growth hormone secretagogue receptor family comprising:

a) introducing at least one nucleic acid suspected of encoding a GHSR or GHSRR into a cell which does not naturally express the receptor on its cell membrane;

b) introducing a G-protein subunit into the cell;

c) introducing a detector molecule or a nucleic acid encoding a detector molecule into the cell, wherein the detector molecule is directly or indirectly responsive to a GHSR-ligand or GHSRR-ligand binding event;

d) contacting the cell with a growth hormone secretagogue; and

e) determining whether the nucleic acid encodes a receptor by monitoring the detector molecule.

A further embodiment of this invention is an assay to determine the presence of a growth hormone secretagogue. Thus, this invention also comprises a method to determine the presence of a growth hormone secretagogue comprising:

a) introducing a nucleic acid which encodes a growth hormone secretagogue receptor into a cell under conditions so that growth hormone secretagogue receptor is expressed;

b) introducing a G-protein subunit into the cell;

c) introducing a detector molecule or a nucleic acid encoding a detector molecule into the cell, wherein the detector molecule is directly or indirectly responsive to a GHSR-ligand binding event;

d) contacting the cell with a compound suspected of being a growth hormone secretagogue; and

e) determining whether the compound is a growth hormone secretagogue by monitoring the detector molecule.

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1 is the DNA of Swine GHSR (Type I) contained in Clone 7-3.

FIGURE 2 is the amino acid sequence of swine GHSR encoded by the DNA of Figure 1.

FIGURE 3 is the entire open reading frame of the Type I clone, of Figure 1.

FIGURE 4 is the DNA of Swine GHSR (Type II) contained in Clone 1375.

FIGURE 5 is the amino acid sequence of swine GHSR (Type II) encoded by the DNA of Figure 4.

FIGURE 6 is the DNA for human GHSR (Type I) contained in Clone 1146.

FIGURE 7 is the amino acid sequence of human GHSR (Type 1) encoded by the DNA of Figure 6.

FIGURE 8 is the entire open reading frame of Type I GHSR, encoded by DNA sequence of Figure 6.

FIGURE 9 is the DNA for human GHSR (Type II) contained in Clone 1141.

FIGURE 10 is the amino acid sequence of human GHSR (Type II) encoded by Clone 1141.

FIGURE 11 is the DNA for human GHSR (Type I) contained in Clone 1143.

FIGURE 12 is the amino acid sequence of human GHSR (Type I) encoded by Clone 1143.

FIGURE 13 compares the ORF of swine Type I (lacking the MET initiator of the full length GHSR and lacking 12 additional amino acids) to the homologous domain of swine Type II receptors.

FIGURE 14 compares the homologous domain of human Type I and Type II receptors (the amino terminal sequence lacks the MET initiator and four additional amino acids).

FIGURE 15 compares the ORFs of swine Type I and human Type I receptors (the amino terminal sequence lacks the MET initiator and 12 additional amino acids).

FIGURE 16 compares full length swine Type II and human Type II receptors.

FIGURE 17 is a schematic diagram depicting the physical map of swine and human growth hormone secretagogue receptor cDNA clones.

FIGURE 18 is a graph demonstrating the pharmacology of the expressed swine and human growth hormone secretagogue receptors in *Xenopus* oocytes using the aequorin bioluminescence assay.

FIGURE 19 is a table demonstrating the pharmacology of the expressed swine and human growth hormone secretagogue receptors in *Xenopus* oocytes using the aequorin bioluminescence assay and various secretagogues.

FIGURE 20 is a graph representing the pharmacology of the pure expressed swine growth hormone secretagogue receptor in COS-7 cells using the ^{35}S-labeled Compound A binding assay.

FIGURE 21 is a table representing the competition analysis with the pure expressed swine growth hormone secretagogue receptor in COS-7 cells using the ^{35}S-labeled Compound A binding assay and various secretagogues and other G-protein coupled- receptors (GPC-receptors) ligands in a competition assay.

FIGURE 22 is the amino acid sequence of the full length human GHSR (Type I) encoded by clone 11304.

FIGURE 23A and 23B are graphs of measurement of [^{35}S]-Compound A binding to swine anterior pituitary membranes. 23A shows results of saturation experiments using a fixed amount of membrane. 23B shows saturation isotherms analyzed by Scatchard analysis.

FIGURE 24 shows the inhibition of [^{35}S]-Compound A binding to porcine anterior pituitary membranes by various compounds.

FIGURE 25 shows the effect of GHRP-6 on specific [^{35}S]-Compound A binding to porcine anterior pituitary membranes at equilibrium.

FIGURE 26 shows the effects of GTP-γ-S and nucleotide on the specific [^{35}S]-Compound A binding to porcine anterior pituitary membranes.

FIGURE 27 is the rat GHSR DNA sequence from the Met Initiation codon to the Stop codon. This sequence includes an intron.

FIGURE 28 is the open reading frame only of the rat GHSR of Figure 27.

FIGURE 29 is the deduced amino acid sequence of the ORF of Figure 28.

FIGURE 30 shows expression of functional rat GHSR in transfected HEK-293 cells.

As used throughout the specification and claims, the following definitions apply:

"Ligands" are any molecule which binds to a GHSR of this invention. Ligands can have either agonist, partial agonist, partial antagonist or antagonist activity.

"Growth hormone secretagogue" or "GHS" is any compound or agent that directly or indirectly stimulates or increases the release of growth hormone in an animal.

"Compound A" is (N-[1(R)-[1,2-dihydro-1-methane-sulfonylspiro[3H-indole-3,4'-piperidin]-1'-yl)carbonyl]-2-(phenyl-methyloxy)-ethyl]-2-amino-2-methyl-propanamide, described in Patchett *et al*, 1995 *Proc. Natl. Acad. Sci* 92: 7001-7005.

"Compound B" is (3-amino-3-methyl-N-(2,3,4.5-tetrahydro-2-oxo-1{2'-(1H-tetrazol-5-yl)(1,1'-biphenyl)-4-yl]-methyl}1H-1-benzazepin-3(R)yl-butanamide, described in Patchett *et al*, 1995 *Proc. Natl. Acad. Sci.* 92: 7001-7005.

This invention relates to assays for members of the growth hormone secretagogue receptor family of proteins, which includes growth hormone secretagogue receptors and growth hormone secretagogue related receptors. The growth hormone secretagogue receptor proteins, growth hormone receptor related proteins, nucleic acids encoding them and methods of making them using genetic engineering techniques are the subject of co-pending United States Provisional Patent Application Nos. 60/008,582, filed December 13, 1995 and (Attorney Docket No. 19589PV2), filed herewith.

The proteins of this invention were found to have structural features which are typical of the 7-transmembrane domain (TM) containing G-protein linked receptor superfamily (GPC-R's or 7-TM receptors) receptors. Thus growth hormone secretagogue receptors make up new members of the GPC-R family of receptors. The intact receptors of this invention were found to have the general features of GPC-R's, including seven transmembrane regions, three intra- and extracellular loops, and the GPC-R protein signature sequence. The transmembrane domains and the GPC-receptor signature sequence are noted in the protein sequences of the Type I GHS receptor in Figures 3 and 8. Not all regions are required for functioning.

The GHSRs share some sequence homology with previously cloned GPC-receptors including the rat and human neurotensin receptor (approximately 32% identity) and the rat and human TRH receptor (approximately 30% identity).

The GHSRs were isolated and characterized using expression cloning techniques in *Xenopus* oocytes. The cloning was made difficult by three factors. First, prior to this invention, there was very little information available about both the biochemical characteristics, and the intracellular signaling/effector pathways of the proteins. Thus, cloning approaches which depend on the use of protein sequence information for the design of degenerate oligonucleotides to screen cDNA libraries or utilize the PCR could not be effectively utilized. Therefore, receptor bioactivity needed to be determined.

Secondly, the growth hormone secretagogue receptor does not occur in abundance-- it is present on the cell membrane in about 10 fold less concentration than most other membrane receptors. In order to successfully clone the receptors, exhaustive precautions had be taken to ensure that the GHSR was represented in a cDNA library to be screened. This required: 1) isolation of intact, undegraded and pure poly (A)+ mRNA; 2) optimization of cDNA synthesis to maximize the production of full-length molecules; and 3) a library of larger size than normal needed to be screened (approximately 0.5 to 1 x 10^7 clones) to increase the probability that a functional cDNA clone may be obtained.

Thirdly, no permanent cell line which expresses these receptors is known. Therefore, primary pituitary tissue had to be used as a source for mRNA or protein. This is an added difficulty because most primary tissues express lower amounts of a given receptor than an immortalized cell line or tumor tissues. Further, the surgical removal of a pig pituitary and extraction of biologically active intact mRNA for the construction of a cDNA expression library is considerably more difficult than the extraction of mRNA from a tissue culture cell line. Along with the need to obtain fresh tissue continuously, there are problems associated with its intrinsic inter-animal and inter-preparation variability.

One aspect of this invention is directed to the development of an extremely sensitive, robust, reliable and high-throughput screening assay which could be used to identify portions of a cDNA library encoding the receptor.

The ability to identify cDNAs which encode growth hormone secretagogue receptors depended upon two discoveries made in accordance with this invention: 1) that growth hormone secretagogue receptor-ligand binding events are transduced through G proteins; and 2) that a particular G protein subunit, such as $G\alpha 11$, must be present in the cells in order to detect receptor activity. Only when these two discoveries were made could an assay be devised to detect the presence of GHSR encoding DNA sequences.

Determination that GHSR is distinct from the Growth Hormone Receptor

A radioreceptor assay using high specific activity (700-1,100 Ci/mmole) [^{35}S]-labeled Compound A (a known GHS) as ligand was developed. Saturable, high affinity binding was detected in porcine anterior pituitary membranes (FIGURE 23A). Scatchard analysis (FIGURE 23B) indicated the presence of a single class of high affinity sites with an apparent dissociation constant (K_D) of 161±11 pM and a concentration (B_{max}) of 6.3±0.6 fmol/mg of protein (n=4). A similar specific high affinity binding was detected in rat pituitary membranes indicating a K_D value of 180±9 pM and B_{max} of 2.3±1.1 fmol/mg protein (n=3).

The high affinity binding to the GHSR makes up yet another aspect of this invention. This invention is also directed to a method of identifying novel GHSR proteins comprising labeling a known ligand, exposing it to a putative GHSR protein and determining if binding occurs.

The specificity of [^{35}S]-Compound A binding was established by determining the ability of GH secretagogues to compete with the radioligand for the binding sites (FIGURE 24). Unlabeled Compound A completely displaced [^{35}S]-Compound A from specific binding sites with an inhibition constant, K_i, of 240 pM which is similar to the K_D value determined by Scatchard analysis. Other GHSs, GHRP-6 (K_i 6.3 nM), and peptide antagonist Compound B (K_i 63 nM) had affinities of 3.8, 0.6 and 0.4%, respectively, of that of Compound A. Compound C, the biologically inactive stereoisomer of Compound B, competed poorly with [^{35}S]-Compound A binding. The saturation isotherm for [^{35}S]-Compound A binding analyzed by double reciprocal plot showed that GHRP-6 inhibition was overcome by increasing concentration of [^{35}S]-Compound A (FIGURE 25). This result shows that GHRP-6 interacts competitively with Compound A in the same binding site. Similarly, Compound B was shown to be a competitor of [^{35}S]-Compound A binding. The most potent agonists had the highest affinities for pituitary receptor sites. Compounds which did not

compete with [^{35}S]-Compound A at 1 μM included GHRH, somatostatin, met-enkephalin, substance P, galanin, gonadotropin releasing hormone, thyrotropin releasing hormone, gastrin releasing peptide, PHM-27, melanocyte stimulating hormone, pituitary adenylate cyclase activating polypeptide-38, phenoxybenzamine, dopamine, bromocriptine, methoxamine, benoxathian, isoproterenol, propanolol and clonidine.

A GHSRR gene may be identified by hybridizing a cDNA encoding a GHSR to a genomic DNA, under relaxed post-hybridizational washing conditions (6 X SSC at 30°C) or moderate post-hybridizational washing conditions (6 X SSC at 45°C). The hybridized area can be identified, isolated and the GHSRR can be cloned and the receptor expressed using conventional techniques.

Determination that GHSR is a G-Protein Receptor

To study whether the [^{35}S]-Compound A specific binding site was G-protein linked, the effects of stable GTP analogs GTP-γ-S and GMP-PNP on [^{35}S]-Compound A binding were studied. GTP-γ-S and GMP-PNP were found to be potent inhibitors of [^{35}S]-Compound A binding with IC_{50} values of 30 and 110 nM, respectively (FIGURE 26). ATP-γ-S was ineffective. In addition, in the absence of Mg^{2+}, only 15-25% of specific binding of [^{35}S]-Compound A binding was detected in comparison with control (10 mM Mg^{2+}) suggesting that the specific binding of [^{35}S]-Compound A required the presence of Mg^{2+} regulate GH release *in vivo*) do not bind to the Compound A site. From these data, one can conclude that the receptor is G-protein linked.

When the GHSR is bound by ligand (a growth hormone secretagogue), the G-proteins present in the cell activate phosphatidylinositol-specific phospholipase C (PI-PLC), an enzyme which releases intracellular signaling molecules (diacylglycerol and inositol tri-phosphate), which in turn start a cascade of biochemical events that promote calcium mobilization. In accordance with this invention, detection of this biochemical cascade can be used as the basis of an assay.

Virtually any convenient eukaryotic cell may be used in the assay of this invention. These would include oocytes (preferred ones are from *Xenopus sp.*) but cell lines may be used as well as Examples of preferred cell lines are mammalian cell lines, including COS, HEK-293, CHO, HeLa, NS/0, CV-1, GC, GH3 and VERO.

One important component of the assay is a detector molecule. Preferably, the detector molecule is responsive to an intracellular event which is part of the biochemical cascade initiated by GHS-GHSR binding. One class of preferred detector molecules can respond to changes in calcium concentrations. A preferred detector molecule which responds to calcium concentrations is aequorin (a jellyfish photoprotein) which acts on the substrate coelenterazine. Other detector molecules include calcium chelators with fluorescence capabilities, such as FURA-2 and indo-1.

The detector molecule itself may be introduced into the cell, or nucleotides which encode the detector molecule may be introduced into the cell, under conditions which will allow the expression of the detector molecule. Generally, it is preferred to introduce nucleotides, such as DNA which encode the detector molecule into the cell, under conditions wherein the cell will express the detector molecule.

Heterotrimeric G proteins, consisting of α, β and γ subunits, serve to relay information from cell surface receptors to intracellular effectors, such as phospholipase C and adenylate cyclase. The G-protein alpha subunit is an essential component of the intracellular signal transduction pathway activated by receptor-ligand interaction. In the process of ligand-induced GPCR activation, the Gα subunit of a trimeric G$\alpha\beta\gamma$ complex will exchange its bound GDP for GTP and dissociate from the $\beta\gamma$ heterodimer. The dissociated Gα-protein serves as the active signal transducer, often in concert with the $\beta\gamma$ complex, thus starting the activation of the intracellular signal transduction pathway. G-alpha subunits are classified into sub-families based on sequence identity and the main type of effectors are coupled:

G_S, activate adenylate cyclase, $G_{i/o/t}$, inhibit adenylate cyclase, $G_q/11$, activate PI-PLC, and G12/13, effector unknown.

The expression of several receptors in heterologous cells has been shown to be increased by the co-expression of certain Gα subunits. This observation formed the basis for the rationale to use Gα subunits of several sub-families in conjunction with a source of GHSR (swine poly A^+ mRNA) to test if a GHS-induced functional response could be measured in the *Xenopus* oocyte system. GHS-induced responses were detected and were found to be strictly dependent on Gα11 co-expression, a unprecedented finding outlining the specificity of the interaction. The finding that the expression of the GPCR could be fully dependent on the addition of a single G-protein subunit was unexpected, since in all previously published work the addition of a G-protein subunit modulated an already existing activity. Here a previously absent signal was fully restored. This finding indicated that the lack of a signal in *Xenopus* eggs was fully dependent on a G-protein subunit as the limiting factor.

In conducting the assay, either the subunit itself or a nucleic acid encoding the subunit, or both may be added, and the addition events need not occur together.

Next, a nucleic acid or pool of nucleic acids, wherein at least one nucleic acid is suspected of encoding a GHSR or GHSRR is introduced into the cell. When trying to identify a possible GHSR or GHSRR gene from a large library, it is often more efficient to use a pool of nucleic acids, each nucleic acid being different from the other nucleic acids in the pool.

After the nucleic acid(s) suspected of encoding a GHSR or GHSRR is introduced into the cell, the cell is exposed to a known growth hormone secretagogue, such as Compound A (L-163,191). Any other growth hormone secretagogue may also be used. Preferred ones include: N-[1(R)-[(1,2-dihydro-1-methanesulfonylspiro[3H-indole-3,4'-piperidin]-1'-yl)carbonyl]-2-(phenylmethyloxy)ethyl]-2-amino-2-methylpropanamide, or 3-amino-3-methyl-N-(2,3,4,5-tetrahydro-2-oxo-1-{[2'-1H-tetrazol-5-yl)(1,1'-biphenyl)-4-yl]methyl}-1H-1-benzazepin-

3(R)-yl-butanamide, or a compound disclosed, for example, in the following: U.S. Patent No. 3,239,345; U.S. Patent No. 4,036,979; U.S. Patent No. 4,411,890; U.S. Patent No. 5,206,235; U.S. Patent No. 5,283,241; U.S. Patent No. 5,284,841; U.S. Patent No. 5,310,737; U.S. Patent No. 5,317,017; U.S. Patent No. 5,374,721; U.S. Patent No. 5,430,144; U.S. Patent No. 5,434,261; U.S. Patent No. 5,438,136; U.S. Patent No. 5,494,919; U.S. Patent No. 5,494,920; U.S. Patent No. 5,492,916; EPO Patent Pub. No. 0,144,230; EPO Patent Pub. No. 0,513,974; PCT Patent Pub. No. WO 94/07486; PCT Patent Pub. No. WO 94/08583; PCT Patent Pub. No. WO 94/11012; PCT Patent Pub. No. WO 94/13696; PCT Patent Pub. No. WO 94/19367; PCT Patent Pub. No. WO 95/03289; PCT Patent Pub. No. WO 95/03290; PCT Patent Pub. No. WO 95/09633; PCT Patent Pub. No. WO 95/11029; PCT Patent Pub. No. WO 95/12598; PCT Patent Pub. No. WO 95/13069; PCT Patent Pub. No. WO 95/14666; PCT Patent Pub. No. WO 95/16675; PCT Patent Pub. No. WO 95/16692; PCT Patent Pub. No. WO 95/17422; PCT Patent Pub. No. WO 95/17423; PCT Patent Pub. No. WO 95/34311; PCT Patent Pub. No. WO 96/02530; *Science*, *260*, 1640-1643 (June 11, 1993); *Ann. Rep. Med. Chem.*, *28*, 177-186 (1993); *Bioorg. Med. Chem. Ltrs.*, *4*(22), 2709-2714 (1994); and *Proc. Natl. Acad. Sci. USA 92*, 7001-7005 (July 1995), or any other growth hormone secretagogue.

If one or more of the nucleic acids does encode a GHSR, or GHSRR, then the secretagogue ligand will bind the receptor, G-protein will be activated, the calcium level will fluctuate, and the detector molecule will change so that it can be monitored. For the system using aequorin and coelenterazine, receptor-GHS binding will produce measurable bioluminescence.

If the procedure used a complex pool of nucleic acids, one or more of which may encode the receptor, then further screening will be necessary to determine which nucleic acid is responsible for encoding GHSR or GHSRR. Once a positive result is found, the procedure can be repeated with a sub-division of the nucleic acid pool (for example,

starting with approximately 10,000 nucleic acids, then using approximately 1,000, then approximately 500, then approximately 50, and then pure). In this procedure, RNA pools are preferred.

Using this general protocol in *Xenopus* oocytes with a swine cDNA expression library, Clone 7-3 was identified as containing nucleic acid encoding a swine growth hormone secretagogue receptor. The clone is approximately 1.5 kb in size, and downstream from the presumed initiator methionine (MET), contains an open reading frame (ORF) encoding 302 amino acids (M_r= 34,516). The DNA and deduced amino acid sequence is given in FIGURES 1 and 2. When hydropathy analysis (e.g. Kyte-Doolittle; Eisenberg, Schwartz, Komaron and Wall) is performed on the protein sequence of clone 7-3, only 6 predicted transmembrane domains are present downstream of the presumed MET initiator. However, translation of the longest ORF encoded in clone 7-3 encodes a protein of 353 amino acids (M_r= 39,787), but is devoid of an apparent initiator MET (FIGURE 3). Seven transmembrane segments are encoded in the longer, 353 amino acid protein in which a MET translation initiation codon located upstream of TM1 is absent (FIGURE 3). Thus, clone 7-3 appears truncated at its amino terminus, but is fully functional, demonstrating that clone 7-3 is a functional equivalent of a native GHSR.

The resultant cDNA clone (or shorter portions of for instance only 15 nucleotides long) may be used to probe libraries under hybridization conditions to find other receptors which are similar enough so that the nucleic acids can hybridize, and is particularly useful for screening libraries from other species. Using this procedure, additional human, swine and rat GHSR cDNAs have been cloned and their nucleotide sequence determined. In this step, one of ordinary skill in the art will appreciate that the hybridization conditions can vary from very stringent to relaxed. Proper temperature, salt concentrations, and buffers are well known. As used herein, "standard post hybridizational washing" conditions mean 6 x SSC at 55°C. "Relaxed post hybridizational washing" conditions means 6 x SSC at 30°C.

A swine pituitary library, a human pituitary library, and a rat pituitary library were hybridized with a radiolabeled cDNA derived from the open reading frame of the swine GHSR clone 7-3. Twenty one positive human GHSR cDNA clones were isolated and five swine library pools yielded a strong hybridization signal and contained clones with inserts larger than clone 7-3, as judged from their insert size on Southern blots. A single rat cDNA clone was also isolated.

Nucleotide sequence analysis revealed two types of cDNAs for both the human and swine GHSR cDNAs. The first (Type I) encodes a protein represented by clone 7-3, encoding 7-TM domains (the amino acid sequence of a full length human clone 11304 is shown in FIGURE 22). The full length open reading frame extends 13 amino acids beyond the largest predicted open reading frame of clone 7-3, (353 amino acids).

The second (type II) diverges in its nucleotide sequence from the type I cDNA at its 3'-end, at the second predicted amino acid of TM-6. In the type II cDNAs, TM-6 is truncated and fused to a short contiguous reading frame of only 24 amino acids, followed by a translation stop codon. Swine clone 1375 is an example of a Type II cDNA (FIGURES 4 and 5). These 24 amino acids beyond TM-6 are highly conserved when compared between human and swine cDNAs. The DNA and amino acid sequences of the human GHSR Type I and II are given in FIGURES 6-12 and 22. A predicted full length cDNA encoding the human Type I receptor, that is, a molecule encoding 7-TM domains with an initiator MET in a favorable context preceded by an inframe termination codon is isolated, and termed clone 11304. The predicted ORF of clone 11304 for the full length Type I GHSR measures 366 amino acids (M_r= 41,198; FIGURE 22). A full length human Type II cDNA encodes a polypeptide of 289 amino acids (M_r=32,156; FIGURES 9 and 10). Sequence alignments performed at both the nucleic acid and protein levels show that Type I and II GHSR's are highly related to each other and across species (FIGURES 13-16). The human and swine GHSR sequences are 93% identical and 98% similar at the amino acid level.

The nucleotide sequence encoding the missing amino terminal extension of swine Type I clone 7-3 is derived from the full length human Type I clone as well as the human and swine Type II cDNAs. The reading frame of the full length clones extended 13 amino acids beyond the amino terminal sequence of clone 7-3 and this sequence was conserved in 12/13 amino acid residues when compared between human and swine. The amino terminal extension includes a translation initiator methionine in a favorable context according to Kosak's rule, with the reading frame further upstream being interrupted by a stop codon. A schematic physical map of Type I and II swine and human cDNA clones is given in FIGURE 17.

The rat clone was also further investigated. Sequence analysis revealed the presence of a non-coding intronic sequence at nt 790 corresponding to a splice-donor site (see FIGURES 27, 28, and 29.) The G/GT splice-donor site occurs two amino acids after the completion of the predicted transmembrane domain 5 (leucine 263), thus dividing the rat GHSR into an amino-terminal segment (containing the extra cellular domain, TM-1 through TM-5, and the first two intra- and extra- cellular loops) and a carboxy-terminal segment (containing TM-6, TM-7, the third intra- and extra- cellular loops, and the intra-cellular domain). The point of insertion and flanking DNA sequences are highly conserved, and also present in both human and swine Type I and II cDNAs.

Comparison of the complete open reading frame encoding the rat GHSR protein to human and swine homologs reveals a high degree of sequence identity (rat vs. human, 95.1%; rat vs. swine 93.4%).

Human and swine Type I cRNAs expressed in oocytes were functional and responded to concentrations Compound A ranging from 1 μM to as low as 0.1 nM in the aequorin bioluminescence assay. Human or swine Type II-derived cRNAs that are truncated in TM-6 failed to give a response when injected into oocytes and these represent a receptor subtype which may bind the GHS, but cannot effectively activate the intracellular signal transduction pathway. In addition the

Type II receptor may interact with other proteins and thus reconstitute a functional GHSR. Proteins such as these which may have ligand-binding activity, but are not active in signal transduction are particularly useful for ligand-binding assays. In these cases, one may also over-express a mutant protein on the cell membrane and test the binding abilities of putative labeled ligands. By using a non-signaling mutant which is constitutively in a high affinity state, binding can be measured, but no adverse metabolic consequences would result. Thus use of non-signaling mutants are an important aspect of this invention.

The pharmacological characterization of human Type I, swine Type I and rat receptors in the aequorin bioluminescence assay in oocytes is summarized in FIGURES 18, 19 and 30. Peptidyl and non-peptidyl bioactive GHS's were active in a similar rank order of potency as observed for the native pituitary receptor. Independent confirmatory evidence that the Type I GHSR (shown for swine clone 7-3) encodes a fully-functional GHSR is given by the finding that when clone 7-3 is expressed transiently in mammalian COS-7 cells, high affinity (KD ~ 0.2 nM), saturable (B_{max}~80 fmol/mg protein) and specific binding (> 90 % displaced by 50 nM unlabeled Compound A) is observed for ^{35}S-Compound A (FIGURES 20-21).

By varying the parameters of the above assays, one can search for other unknowns. For example, in the assay which detects whether a nucleic acid which encodes a GHSR or GHSRR is present, one can modify the assay so that it detects whether a GHS is present. In this embodiment, a nucleic acid encoding GHSR or GHSRR is introduced into the cell, as well as a nucleic acid encoding a detector molecule, and a G protein subunit. The cell is contacted with at least one compound which is a putative GHS. If the compound is a GHS, then the GHS will bind the GHSR or GHSRR, and the resultant intracellular events can be detected by monitoring the detector molecule. If the compound is not a GHS, then no such activity will be detected. This GHS assay forms yet another aspect of this invention.

A further aspect of this invention are novel ligands which are identified using the above assay.

Expression of several receptors in heterologous cells has been shown to be increased by the co-expression of certain Gα subunits. This observation formed the basis for the rationale to the use of Gα subunits of several sub-families in conjunction with a source of GHSR (swine poly[A+] mRNA) to test if a GHS-induced functional response could be measured in the *Xenopus* oocyte system. GHS-induced responses were detected and were found to be strictly dependent on Gα11 co-expression, an unprecedented finding outlining the specificity of the interaction. Thus another aspect of this invention is a method of detecting a GHS response comprising co-expressing a Gα11 protein subunit in a cell also expressing a GHSR, exposing the cell to a GHS, and detecting the response.

The presence of Gα11 was essential in using poly A+ RNA or complex cRNA pools (i.e. 10.000 cRNAs). However, once a pure clone was obtained the requirement for the G-protein addition was no longer essential. This indicates that the need for G-protein addition depended on the purity of the nucleic acid, the most sensitive assay requiring Gα subunit addition. Thus another aspect of this invention is a method of determining the presence of an nucleic acid which encodes a growth hormone secretagogue receptor or growth hormone secretagogue related receptor comprising:

a) introducing a nucleic acid suspected of encoding a GHSR or GHSRR into a cell which does not naturally express the receptor on its cell membrane;

b) introducing a detector molecule or a nucleic acid encoding a detector molecule into the cell, wherein the detector molecule is directly or indirectly responsive to a receptor-ligand binding event;

c) contacting the cell with a growth hormone secretagogue; and

d) determining whether the nucleic acid encodes a receptor by monitoring the detector molecule.

Similarly, another aspect of this invention is an assay method to determine the presence of a growth hormone secretagogue comprising:

a) introducing a nucleic acid which encodes a growth hormone secretagogue receptor into a cell under conditions so that growth hormone secretagogue receptor is expressed;

b) introducing a detector molecule or a nucleic acid encoding a detector molecule into the cell, wherein the detector molecule is directly or indirectly responsive to a GHSR-ligand binding event;

c) contacting the cell with a compound suspected of being a growth hormone secretagogue; and

d) determining whether the compound is a growth hormone secretagogue by monitoring the detector molecule.

Ligands detected using assays described herein may be used in the treatment of conditions which occur when there is a shortage of growth hormone, such as observed in growth hormone deficient children, elderly patients with musculoskeletal impairment and recovering from hip fracture, patients with neurodegenerative diseases, and patients recovering from coronary by-pass surgery, and osteoporosis.

A GHS receptor, preferably imobilized on a solid support, may be used diagnostically for the determination of the concentration of growth hormone secretagogues, or metabolites thereof, in physiological fluids, e.g., body fluids, including serum, and tissue extracts, as for example in patients who are undergoing therapy with a growth hormone secretagogue.

The administration of a GHS receptor to a patient may also be employed for purposes of: amplifying the net effect of a growth hormone secretagogue by providing increased downstream signal following administration of the growth hormone secretagogue thereby

diminishing the required dosage of growth hormone secretagogue; or diminishing the effect of an overdosage of a growth hormone secretagogue during therapy.

The following non-limiting Examples are presented to better illustrate the invention.

EXAMPLE 1

Preparation of High Specific Activity Radioligand [^{35}S]-Compound A-

[^{35}S]-Compound A was prepared from an appropriate precursor, N-[1(R)-[(1,2-dihydrospiro[3H-indole-3,4'-piperidin]-1'-yl)-carbonyl]-2-(phenyl-methyloxy)ethyl]-2-amino-t-butoxycarbonyl-2-methylpropanamide, using methane [^{35}S]sulfonyl chloride as described in Dean DC, *et al.*, 1995, In: Allen J, Voges R (eds) Synthesis and Applications of Isotopically Labelled Compounds, John Wiley & Sons, New York, pp. 795-801, Purification by semi-preparative HPLC (Zorbax SB-phenyl column, 68% MeOH/water, 0.1% TFA, 5 ml/min) was followed by N-t-BOC cleavage using 15% trifluroacetic acid in dichloromethane (25°C, 3 hr) to give [methylsulfonyl-^{35}S]Compound A in near quantitative yield. HPLC purification (Hamilton PRP-1 4.6x250 mm column, linear gradient of 50-75% methanol-water with 1 mM HCl over 30 min, 1.3 ml/min) provided the ligand in >99% radiochemical purity. The structure was established by HPLC coelution with unlabeled Compound A and by mass spectral analysis. The latter method also indicated a specific activity of ~1000 Ci/mmol.

EXAMPLE 2

Preparation of Pituitary Membranes

Frozen anterior pituitary glands from male swine (50-80 Kg) or from the Wistar male rats (150-200 g) were homogenized in a tissue homogenizer in ice-cold buffer (50 mM Tris-HCl buffer, pH 7.4, 5 mM $MgCl_2$, 2.5 mM EDTA, 0.1% bovine serum albumin and 30 µg/ml bacitracin). The homogenates were centrifuged for 5 min at

1,400 xg and the resulting supernatants were then centrifuged at 34,000 xg for 20 min. The pellets were resuspended in same buffer to a 1,500 µg protein/ml and stored at -80°C. Protein was determined by a Bio-Rad method (Bio-Rad Laboratories, Richmond, CA).

EXAMPLE 3

Receptor Binding Assay

The standard binding solution contained: 400 m of 25 mM Tris-HCl buffer, pH 7.4, 10 mM $MgCl_2$, 2.5 mM EDTA, and 100 pM [^{35}S]-Compound A. Pituitary membranes (100 µl, 150 µg protein) were added to initiate the binding reaction. Aliquots were incubated at 20°C for 60 min and bound radioligand was separated from free by filtration through GF/C filters pretreated with 0.5% of polyethylenimine in a Brandel cell harvester. The filters were washed three times with 3-ml of ice-cold buffer (50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 2.5 mM EDTA and 0.015% Triton X-100) and the radioactivity on the filters were counted in Aquasol 2. Specific binding was defined as the difference between total binding and nonspecific binding assayed in 500 nM unlabeled Compound A. Specific bindings were 65-85 and 45-60% of total binding, in porcine and rat membranes, respectively. Assays were carried out in triplicate and experiments repeated at least three times.

EXAMPLE 4

Oocyte Preparation and Selection

Xenopus laevis oocytes were isolated and injected using standard methods previously described by Arena, *et. al.* 1991, *Mol. Pharmacol.* 40, 368-374, which is hereby incorporated by reference. Adult female *Xenopus laevis* frogs (purchased from Xenopus One, Ann Arbor, MI) were anesthetized with 0.17% tricaine methanesulfonate and the ovaries were surgically removed and placed in a 60 mm culture dish (Falcon) containing OR-2 medium without calcium (82.5 mM NaCl, 2

mM KCl, 2.5 mM sodium pyruvate, 1 mM $MgCl_2$, 100 µ/ml penicillin, 1 mg/ml streptomycin, 5 mM HEPES, pH=7.5; ND-96 from Specialty Media, NJ). Ovarian lobes were broken open, rinsed several times, and oocytes were released from their sacs by collagenase A digestion (Boehringer-Mannheim; 0.2% for 2-3 hours at 18°C) in calcium-free OR-2. When approximately 50% of the follicular layers were removed, Stage V and VI oocytes were selected and placed in ND-86 with calcium (86 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 2.5 mM sodium pyruvate, 0.5 mM theopylline, 0.1 mM gentamycin, 5 mM HEPES [pH=7.5]). For each round of injection, typically 3-5 frogs were pre-tested for their ability to express a control G-protein linked receptor (human gonadotropin-releasing hormone receptor) and show a robust phospholipase C intracellular signaling pathway (incubation with 1% chicken serum which promotes calcium mobilization by activation of phospholipase C). Based on these results, 1-2 frogs were chosen for library pool injection (50 nl of cRNA at a concentration of 25 ng (complex pools) to 0.5 ng (pure clone) per oocyte usually 24 to 48 hours following oocyte isolation.

EXAMPLE 5

mRNA Isolation

Total RNA from swine (50-80 kg, Yorkshire strain) pituitaries (snap-frozen in liquid nitrogen within 1-2 minutes of animal sacrifice) was prepared by a modified phenol:guanidinium thiocyanate procedure (Chomczynski, *et al*, 1987 *Anal. Biochem.* 162, 156-159, which is hereby incorporated by reference), using the TRI-Reagent LS as per the manufacturer's instructions (Molecular Research Center, Cincinnati, OH). Typically, 5 mg of total RNA was obtained from 3.5 g wet weight of pituitary tissue. Poly $(A)^+$ RNA was isolated from total RNA by column chromatography (two passes) on oligo (dT) cellulose (Pharmacia, Piscataway, NJ). The yield of poly $(A)^+$ mRNA from total RNA was usually 0.5%. RNA from other tissues was isolated similarly.

EXAMPLE 6

cDNA Library Construction

First-strand cDNA was synthesized from poly (A)$^+$ mRNA using M-MLV RNAse (-) reverse transcriptase (Superscript, GIBCO-BRL, Gaithersberg, MD) as per the manufacturer's instructions with an oligo (dT)/Not I primer-adapter. Following second-strand cDNA synthesis, double-stranded cDNA was subjected to the following steps: 1) ligation to EcoR I adapters, 2) Not I digestion, and 3) enrichment for large cDNAs and removal of excess adapters by gel filtration chromatography on a Sephacryl S-500 column (Pharmacia). Fractions corresponding to high molecular weight cDNA were ligated to EcoR I/Not I digested pSV-7, a eucaryotic expression vector capable of expressing cloned cDNA in mammalian cells by transfection (driven by SV-40 promoter) and in oocytes using *in vitro* transcripts (initiated from the T7 RNA polymerase promoter). pSV-7 was constructed by replacing the multiple cloning site in pSG-5 (Stratagene, La Jolla, CA; Green, S. *et al*, 1988 *Nucleic Acids Res.* 16:369, which is hereby incorporated by reference) with an expanded multiple cloning site. Ligated vector:cDNA was transformed into *E.coli* strain DH10B (GIBCO-BRL) by electroporation with a transformation efficiency of 1 x 10^6 pfu/10 ng double-stranded cDNA. The library contained approximately 3 x 10^6 independent clones with greater than 95% having inserts with an average size approximating 1.65 kb (range 0.8-2.8 kb). Unamplified library stocks were frozen in glycerol at -70°C until needed. Aliquots of the library were amplified once prior to screening by a modification of a solid-state method (Kriegler, M. in *Gene Transfer and Expression: A Laboratory Manual* Stockton Press, NY 1990). Library stocks were titered on LB plates and then the equivalent of 500-1000 colonies was added to 13 ml of 2 x YT media containing 0.3% agarose and 100 µg/ml carbenicillin in a 14 ml round-bottom polypropylene tube (Falcon). The bacterial suspension was chilled in a wet ice bath for 1 hour to solidify the suspension, and then grown upright at 37°C for 24 hrs. The resultant bacterial colonies were

harvested by centrifugation at 2000 x g at RT for 10 min, resuspended in 3 ml 2X YT/ carbenicillin. Aliquots were taken for frozen stocks (5%) and plasmid DNA preparation.

EXAMPLE 7

Plasmid DNA Preparation and cRNA Transcription

Plasmid DNA was purified from pellets of solid-state grown bacteria (1000 pools of 500 independent clones each) using the Wizard Miniprep kit according to the manufacturer's instructions (Promega Biotech, Madison, WI). The yield of plasmid DNA from a 14 ml solid-state amplification was 5-10 µg. In preparation for cRNA synthesis, 4 µg of DNA was digested with Not I, and the subsequent linearized DNA was made protein and RNase-free by proteinase K treatment (10 µg for 1 hour at 37°C), followed by two phenol, two chloroform/isoamyl alcohol extractions, and two ethanol precipitations. The DNA was resuspended in approximately 15 µl of RNase-free water and stored at -70°C until needed. cRNA was synthesized using a kit from Promega Biotech with modifications. Each 50 µl reaction contained: 5 µl of linearized plasmid (approximately 1 µg), 40 mM Tris-HCl (pH=7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 0.05 mg/ml bovine serum albumin, 2 units/ml RNasin, 800 µM each of ATP, CTP and UTP, 200 µM GTP, 800 µM m7G(5')ppp(5')G, 80 units of T7 RNA polymerase, and approximately 20,000 cpm of ^{32}P-CTP as a trace for quantitation of synthesized RNA by TCA precipitation. The reaction was incubated for 3 hrs. at 30°C; 20 units of RNase-free DNase was added, and the incubation was allowed to proceed for an additional 15 min. at 37°C. cRNA was purified by two phenol, chloroform/isoamyl alcohol extractions, two ethanol precipitations, and resuspended at a concentration of 500 ng/ml in RNase-free water immediately before use.

EXAMPLE 8

Aequorin Bioluminescence Assay (ABA) and Clone Identification

The ABA requires injection of library pool cRNA (25 ng/egg for pool sizes of 500 to 10,000) with aequorin cRNA (2 ng/egg) supplemented with the G-protein alpha subunit $G\alpha 11$ (2 ng/egg). To facilitate stabilization of synthetic transcripts from aequorin and $G\alpha 11$ plasmids, the expression vector pCDNA-3 was modified (termed pcDNA-3v2) by insertion (in the Apa I restriction enzyme site of the polylinker) of a cassette to append a poly (A) tract on all cRNA's which initiate from the T7 RNA polymerase promoter. This cassette includes (5' to 3'): a Bgl II site, pA (20) and a Sfi I site which can be used for plasmid linearization. Polymerase chain reaction (PCR) was utilized to generate a DNA fragment corresponding to the open reading frame (ORF) of the aequorin cDNA with an optimized Kosak translational initiation sequence (Inouye, S. *et. al.*, 1985, *Proc. Natl. Acad. Sci. USA* 82:3154-3158). This DNA was ligated into pCDNA-3v2 linearized with EcoR I and Kpn I in the EcoR I/Kpn I site of pCDNA-3v2. $G\alpha 11$ cDNA was excised as a Cla I/Not I fragment from the pCMV-5 vector (Woon, C. *et. al.*, 1989 *J. Biol. Chem.* 264: 5687-93), made blunt with Klenow DNA polymerase and inserted into the EcoR V site of pcDNA-3v2. cRNA was injected into oocytes using the motorized "Nanoject" injector (Drummond Sci. Co., Broomall, PA.) in a volume of 50 nl. Injection needles were pulled in a single step using a Flaming/Brown micropipette puller, Model P-87 (Sutter Instrument Co) and the tips were broken using 53X magnification such that an acute angle was generated with the outside diameter of the needle being <3 μm. Following injection, oocytes were incubated in ND-96 medium, with gentle orbital shaking at 18°C in the dark. Oocytes were incubated for 24 to 48 hours (depending on the experiment and the time required for expression of the heterologous RNA) before "charging" the expressed aequorin with the essential chromophore coelenterazine. Oocytes were "charged" with coelenterazine by transferring them into 35 mm dishes containing 3 ml charging medium and incubating for 2-3 hours with gentle orbital shaking in the dark at 18°C. The charging medium

contained 10 µM coelenterazine (Molecular Probes, Inc., Eugene, OR.) and 30 µM reduced glutathione in OR-2 media (no calcium). Oocytes were then returned to ND-86 medium with calcium medium described above and incubation continued in the dark with orbital shaking until bioluminescence measurements were initiated. Measurement of GHSR expression in oocytes was performed using a Berthold Luminometer LB953 (Wallac Inc., Gaithersburg, MD) connected to a PC running the Autolumat-PC Control software (Wallac Inc., Gaithersburg, MD). Oocytes (singly or in pairs) were transferred to plastic tubes (75 x 12 mm, Sarstedt) containing 2.9 ml Ca^{++}-free OR-2 medium. Each cRNA pool was tested using a minimum of 3 tubes containing oocytes. Bioluminescence measurements were triggered by the injection of 0.1 ml of 30 µM Compound A (1 µM final concentation) and recordings were followed for 2 min. to observe kinetic responses consistent with an IP3-mediated response.

Pool S10-20 was prepared from the unfractionated swine pituitary cDNA library and was composed of 10 pools each of 1000 clones. S10-20 gave a positive signal on two luminometer instruments and the component pools were then individually tested for activity. From the 10 pools of 1000 clones, only pool S271 gave a positive response. This pool was made from two pools of 500 clones designated P541 and P542. Again, only one of the pools, P541, gave a positive bioluminescent signal in the presence of 1 µM Compound A. At this point, the bacterial titer was determined in the glycerol stock of P541 such that dilutions could be plated onto LB agar plates containing 100 µg/ml carbenicillin to yield approximately 50 colonies per plate. A total of 1527 colonies were picked and replicated from 34 plates. The colonies on the original plates were then washed off, plasmids isolated, cRNA synthesized and injected into oocytes. cRNA prepared from 8 of the 34 plates gave positive signals in oocytes. Two plates were selected and the individual colonies from these plates were grown up, plasmid isolated, cRNA prepared and injected into oocytes. A single clonal isolate from each plate (designated as clones 7-3 and 28-18) gave a

positive bioluminescence response to 1 μM Compound A. Clone 7-3 was further characterized.

EXAMPLE 9

Receptor Characterization

DNA sequencing was performed on both strands using an automated Applied Biosystems instrument (ABI model 373) and manually by the dideoxy chain termination method using Sequenase II (US Biochemical, Cleveland, OH). Database searches (Genbank 88, EMBL 42, Swiss-Prot 31, PIR 40, dEST, Prosite, dbGPCR), sequence alignments and analysis of the GHSR nucleotide and protein sequences were carried out using the GCG Sequence Analysis Software Package (Madison, WI; pileup, peptide structure and motif programs), FASTA and BLAST search programs, and the PC/Gene software suite from Intelligenetics (San Francisco, CA; protein analysis programs). Northern blot analysis was conducted using total (20 μg/lane) or poly (A)+ mRNA (5-10 μg/lane) prepared as described above. RNA was fractionated on a 1% agarose gel containing 2.2 M formaldehyde and blotted to a nitrocellulose membrane. Blots were hybridized with a PCR generated probe encompassing the majority of the ORF predicted by clone 7-3 (nt 291 to 1132). The probe was radiolabeled by random-priming with $[\alpha]^{32}$P-dCTP to a specific activity of greater than 10^9 dpm/μg. Blots were pre-hybridized at 42°C for 4 hrs. in 5 X SSC, 5 X Denhardt's solution, 250 μg/ml tRNA, 1% glycine, 0.075% SDS, 50 mM $NaPO_4$ (pH 6) and 50% formamide. Hybridizations were carried out at 42°C for 20 hrs. in 5 X SSC, 1 X Denhardt's solution, 0.1% SDS, 50 mM $NaPO_4$, and 50% formamide. RNA blots were washed in 2 X SSC, 0.2% SDS at 42°C and at -70°C. RNA size markers were 28S and 18S rRNA and *in vitro* transcribed RNA markers (Novagen). Nylon membranes containing EcoR I and Hind III digested genomic DNA from several species (Clontech; 10 mg/lane) were hybridized for 24 hrs. at 30°C in 6 X SSPE, 10 X Denhardt's, 1% SDS, and 50% formamide. Genomic blots were washed twice with room temperature 6 X SSPE, twice with 55°C 6 X SSPE, and twice with 55°C 4 X SSPE. Additional

swine GHSR clones from the swine cDNA library (described above) were identified by hybridization to plasmid DNA (in pools of 500 clones each) immobilized to nylon membranes in a slot-blot apparatus (Scheicher and Schuell). Pure clonal isolates were subsequently identified by colony hybridization. Swine GHSR clones that extend further in a 5' direction were identified using 5' RACE procedures (Frohman, M. A., 1993 *Methods Enzymol.* 218:340-358, which is incorporated by reference) using swine pituitary poly $(A)^+$ mRNA as template.

EXAMPLE 10

Human GHSR

Human pituitary homologues of the swine GHSR were obtained by screening a commercially available cDNA library constructed in the vector lambda ZAP II (Stratagene) as per the manufacturer's instructions. Approximately 1.86 x 10^6 phages were initially plated and screened using a random-primer labeled portion of swine clone 7-3 (described above) as hybridization probe. Twenty one positive clones were plaque purified. The inserts from these clones were excised from the bacteriophage into the phagemid pBluescript II SK- by co-infection with helper phage as described by the manufacturer (Stratagene). Human clones were characterized as has been described above for the swine clone.

EXAMPLE 11

DNA Encoding a Rat Growth Hormone Secretagogue Receptor (GHSR) Type Ia

Cross-hybridization under reduced stringency was the strategy utilized to isolate the rat GHSR type Ia. Approximately 10^6 phage plaques of a once-amplified rat pituitary cDNA library in lambda gt11 (RL1051b; Clontech, Palo Alto, CA) were plated on *E. coli* strain Y1090r$^-$. The plaques were transferred to maximum-strength Nytran

(Schleicher & Schuell, Keene, NH) denatured, neutralized and screened with a 1.6 kb EcoRI/NotI fragment containing the entire coding and untranslated regions of the swine GHSR, clone 7-3. The membranes were incubated at 30°C in prehybridization solution (50% formamide, 2 X Denhardts, 5 X SSPE, 0.1% SDS, 100 µg/ml salmon sperm DNA) for 3 hours followed by overnight incubation in hybridization solution (50% formamide, 2 X Denhardts, 5 X SSPE, 0.1% SDS, 10% dextran sulfate, 100 µg/ml salmon sperm DNA) with 1 x 10^6 cpm/ml of [^{32}P]-labeled probe. The probe was labeled with [^{32}P]dCTP using a random priming kit (Gibco BRL, Gaithersburg, ND). After hybridization the blots were washed two times each with 2 X SSC, 0.1% SDS (at 24°C, then 37°C, and finally 55°C). A single positive clone was isolated following three rounds of plaque purification. Phage containing the GHSR was eluted from plate plaques with 1x lambda buffer (0.1M NaCl, 0.01M $MgSO_4 \cdot 7H_2O$, 35mM Tris-HCl, pH 7.5) following overnight growth of approximately 200 pfu/150mm dish. After a ten minute centrifugation at 10,000 x/g to remove debris, the phage solution was treated with 1µg/ml RNAse A and DNAse I for thirty minutes at 24°C, followed by precipitation with 20% PEG (8000)/2M NaCl for two hours on ice, and collection by centrifugation at 10,000 x/g for twenty minutes. Phage DNA was isolated by incubation in 0.1% SDS, 30mM EDTA, 50 µg/ml proteinase K for one hour at 68°C, with subsequent phenol (three times) and chloroform (twice) extraction before isopropanol precipitation overnight. The GHSR DNA insert (~6.4 kb) was sub-cloned from lambda gt11 into the plasmid vector Litmus 28 (New England Biolabs, Beverly, MA). 2 µg of phage DNA was heated to 65°C for ten minutes, then digested with 100 units BsiWI (New England Biolab, Bevely, MA) at 37°C overnight. A 6.5 kb fragment was gel purified, electroeluted and phenol/chloroform extracted prior to ligation to BsiWI-digested Litmus 28 vector.

Double-stranded DNA was sequenced on both strands on a ABI 373 automated sequencer using the ABI PRISM dye termination cycle sequencing ready reaction kit (Perkin Elmer; Foster City, CA).

For sequence comparisons and functional expression studies, a contiguous DNA fragment encoding the complete ORF (devoid of intervening sequence) for the rat GHSR type Ia was generated. The PCR was utilized to synthesize a amino-terminal fragment from Met-1 to Val-260 with EcoRI (5') and HpaI (3') restriction sites appended, while a carboxyl-terminal fragment was generated from Lys-261 to Thr-364 with Dra I (5') and Not I (3') restriction sites appended. The ORF construct was assembled into the mammalian expression vector pSV7 via a three-way ligation with EcoRI/Not I-digested pSV7, EcoRI/Hpa I-digested NH_2-terminal fragment, and Dra I/Not I-digested C-terminal fragment.

Functional activity of the ORF construct was assessed by transfecting (using lipofectamine; GIBCO/BRL) 5 µg of plasmid DNA into the aequorin expressing reporter cell line (293-AEQ17) cultured in 60 mm dishes. Following approximately 40 hours of expression the aequorin in the cells was charged for 2 hours with coelenterazine, the cells were harvested, washed and pelleted by low speed centrifugation into luminometer tubes. Functional activity was determined by measuring Compound A dependent mobilization of intracellular calcium and concommitant calcium induced aequorin bioluminescence. Shown in Fig. 26 are three replicate samples exhibiting Compound A induced luminescent responses.

EXAMPLE 12

Assays

Mammalian cells (COS-7) were transfected with GHSR expression plasmids using Lipofectamine (GIBCO-BRL: Hawley-Nelson, 1993, *Focus* 15:73). Transfections were performed in 60 mm dishes on 80% confluent cells (approximately 4 x 10^5cells) with 8 µg of Lipofectamine and 32 µg of GHSR plasmid DNA.

Binding of [^{35}S]-Compound A to swine pituitary membranes and crude membranes prepared from COS-7 cells

transfected with GHSR expression plasmids was conducted. Crude cell membranes from COS-7 transfectants were prepared on ice, 48 hrs. post-transfection. Each 60 mm dish was washed twice with 3 ml of PBS, once with 1 ml homogenization buffer (50 mM Tris-HCl [pH 7.4], 5 mM $MgCl_2$, 2.5 mM EDTA, 30 µg/ml bacitracin). 0.5 ml of homogenization buffer was added to each dish, cells were removed by scraping and then homogenized using a Polytron device (Brinkmann, Syosset, NY; 3 bursts of 10 sec. at setting 4). The homogenate was then centrifuged for 20 min. at 11,000 x g at 0°C and the resulting crude membrane pellet (chiefly containing cell membranes and nuclei) was resuspended in homogenization buffer supplemented with 0.06% BSA (0.1 ml/60 mm dish) and kept on ice. Binding reactions were performed at 20°C for 1 hr. in a total volume of 0.5 ml containing: 0.1 ml of membrane suspension, 10 µl of [^{35}S]-Compound A (0.05 to 1 nM; specific activity approximately 900 Ci/mmol), 10 µl of competing drug and 380-390 µl of homogenization buffer. Bound radioligand was separated by rapid vacuum filtration (Brandel 48-well cell harvester) through GF/C filters pretreated for 1 hr. with 0.5% polyethylenimine. After application of the membrane suspension to the filter, the filters were washed 3 times with 3 ml each of ice cold 50 mM Tris-HCl [pH 7.4], 10 mM $MgCl_2$, 2.5 mM EDTA and 0.015% Triton X-100, and the bound radioactivity on the filers was quantitated by scintillation counting. Specific binding (> 90% of total) is defined as the difference between total binding and non-specific binding conducted in the presence of 50 nM unlabeled Compound A.

WHAT IS CLAIMED IS

1. A method of determining the presence of an nucleic acid which encodes a growth hormone secretagogue receptor (GHSR) or a growth hormone related receptor (GHSRR) comprising:

a) introducing a nucleic acid suspected of encoding a GHSR or GHSRR into a cell which does not naturally express the receptor on its cell membrane;

b) introducing a detector molecule or a nucleic acid encoding a detector molecule into the cell, wherein the detector molecule is directly or indirectly responsive to a receptor-ligand binding event;

c) contacting the cell with a growth hormone secretagogue; and

d) determining whether the nucleic acid encodes a receptor by monitoring the detector molecule.

2. A method to determine the presence of a growth hormone secretagogue comprising:

a) introducing a nucleic acid which encodes a growth hormone secretagogue receptor into a cell under conditions so that growth hormone secretagogue receptor is expressed;

b) introducing a detector molecule or a nucleic acid encoding a detector molecule into the cell, wherein the detector molecule is directly or indirectly responsive to a GHSR-ligand binding event;

c) contacting the cell with a compound suspected of being a growth hormone secretagogue; and

d) determining whether the compound is a growth hormone secretagogue by monitoring the detector molecule.

3. A method to determine the presence of a nucleic acid which encodes a G protein cell membrane receptor comprising:

a) introducing at least one nucleic acid suspected of encoding a G protein cell membrane receptor into a cell;

b) introducing a G-protein subunit into the cell;

c) introducing a detector molecule or a nucleic acid encoding a detector molecule into the cell, wherein the detector molecule responds directly or indirectly to a G-protein receptor-ligand binding event;

d) contacting the cell with a receptor ligand; and

e) determining whether the nucleic acid encodes a receptor by monitoring the detector molecule.

4. A method according to Claim 3 wherein the cell does not naturally express the receptor on its cell membrane.

5. A method according to Claim 4 wherein the receptor is a growth hormone secretagogue receptor (GHSR).

6. A method according to Claim 4 wherein the receptor is a growth hormone secretagogue related receptor (GHSRR).

7. An assay to determine the presence of an nucleic acid which encodes a growth hormone secretagogue receptor (GHSR) or a growth hormone secretagogue related receptor (GHSRR) comprising:

a) introducing at least one nucleic acid suspected of encoding a GHSR or GHSRR into a cell which does not naturally express the receptor on its cell membrane;

b) introducing a G-protein subunit into the cell;

c) introducing a detector molecule or a nucleic acid encoding a detector molecule into the cell, wherein the detector molecule is directly or indirectly responsive to a receptor-ligand binding event;

d) contacting the cell with a growth hormone secretagogue; and

e) determining whether the nucleic acid encodes a receptor by monitoring the detector molecule.

8. A method according to Claim 7 wherein a pool comprising at least 500 different nucleic acid molecules are introduced into the cell in step a).

9. A method according to Claim 8 wherein the pool comprises RNA molecules.

10. A method according to Claim 8 wherein upon determining that the pool comprises a nucleic acid encoding a growth hormone secretagogoue recptor is present, steps a) to e) are repeated, except that the pool comprises a smaller number of different nucleic acid molecules.

11. A method according to Claim 10 wherein only one type of oligonucleotide is introduced into the cell in step a).

12. A method according to Claim 7 wherein the G protein subunit is a G-alpha protein subunit.

13. A method according to Claim 12 wherein the G-protein subunit is the $G_{\alpha 11}$ subunit.

14. A method according to Claim 7 wherein the detector molecule is aequorin.

15. An assay method to determine the presence of a growth hormone secretagogue comprising:

a) introducing a nucleic acid which encodes a growth hormone secretagogue receptor into a cell under conditions so that growth hormone secretagogue receptor is expressed;

b) introducing a G-protein subunit into the cell;

c) introducing a detector molecule or a nucleic acid encoding a detector molecule into the cell, wherein the detector molecule is directly or indirectly responsive to a GHSR-ligand binding event;

d) contacting the cell with a compound suspected of being a growth hormone secretagogue; and

e) determining whether the compound is a growth hormone secretagogue by monitoring the detector molecule.

16. A method according to Claim 15 wherein the G protein subunit is an G-alpha protein subunit.

17. A method according to Claim 16 wherein the G-protein subunit is the $G_{\alpha 11}$ subunit.

18. A method according to Claim 15 wherein the result of step e) is compared to that obtained using a known growth hormone secretagogue.

19. An assay for identifying a ligand which binds to a human growth hormone secretagogue receptor comprising contacting a putative ligand with a human growth hormone secretagogue receptor in the presence of G protein subunit $\alpha 11$ and determining whether binding has occurred.

20. An assay according to Claim 19 wherein the human growth hormone secretagogue receptor is expressed in a host cell which does not naturally express human growth hormone secretagogue receptor.

21. An assay according to Claim 20 wherein binding is detected by measuring the activity of a detector molecule.

22. An assay according to Claim 21 wherein the detector molecule is aequorin.

23. A ligand identified by the assay of any of Claims 15-22.

24. A method of identifying a novel GHSR protein comprising exposing a labeled ligand to a putative GHSR protein and determining if binding occurs.

```
          10         20         30         40
          .          .          .          .
CCTCACGCTGCCAGACCTGGGCTGGGACGCTCCCCCTGAA   40
AACGACTCGCTAGTGGAGGAGCTGCTGCCGCTCTTCCCCA   80
CGCCGCTGTTGGCGGGCGTCACCGCCACCTGCGTGGCGCT  120
CTTCGTGGTGGGTATCGCGGGCAACCTGCTCACGATGCTG  160
GTAGTGTCACGCTTCCGCGAGATGCGCACCACCACCAACC  200

          210        220        230        240
          .          .          .          .
TCTACCTGTCCAGCATGGCCTTCTCCGACCTACTCATCTT  240
CCTCTGCATGCCCCTCGACCTCTTCCGCCTCTGGCAGTAC  280
CGGCCTTGGAACCTTGGCAACCTGCTCTGCAAACTCTTCC  320
AGTTCGTTAGCGAGAGCTGCACCTACGCCACAGTGCTCAC  360
CATCACCGCGCTGAGCGTCGAGCGCTACTTCGCCATCTGC  400

          410        420        430        440
          .          .          .          .
TTCCCGCTGCGGGCCAAGGTAGTGGTCACCAAGGGCCGGG  440
TAAAGCTGGTCATCCTGGTCATCTGGGCCGTGGCCTTCTG  480
CAGCGCCGGGCCCATCTTCGTGCTGGTCGGAGTGGAGCAT  520
GATAACGGCACTGACCCTCGGGACACCAACGAGTGCCGCG  560
CCACGGAGTTCGCCGTGCGCTCCGGGCTGCTTACCGTCAT  600

          610        620        630        640
          .          .          .          .
GGTCTGGGTGTCCAGTGTCTTCTTCTTCCTGCCTGTCTTC  640
TGCCTCACTGTGCTCTATAGCCTCATCGGCAGGAAGCTCT  680
GGCGGAGGAAGCGCGGCGAGGCGGCGGTGGGCTCCTCGCT  720
CAGGGACCAGAACCACAAACAAACCGTGAAAATGCTGGCT  760
GTAGTGGTGTTTGCTTTCATACTCTGCTGGCTGCCTTTCC  800

          810        820        830        840
          .          .          .          .
ATGTAGGGCGATATTTATTTTCCAAATCCTTGGAGCCTGG  840
CTCTGTGGAGATTGCTCAGATCAGCCAATACTGCAACCTC  880
GTGTCCTTTGTCCTCTTCTACCTCAGTGCGGCCATCAACC  920
CTATTCTGTACAACATCATGTCCAAGAAGTATCGGGTGGC  960
GGTGTTCAAACTGCTGGGATTTGAGCCCTTCTCACAGAGG 1000

          1010       1020       1030       1040
          .          .          .          .
AAACTCTCCACTCTGAAGGATGAAAGTTCTCGGGCCTGGA 1040
CAGAATCTAGTATTAATACATGA 1063
```

FIG.1

```
          10         20
          .          .
MLVVSRFREM RTTTNLYLSS   20
MAFSDLLIFL CMPLDLFRLW   40
QYRPWNLGNL LCKLFQFVSE   60
SCTYATVLTI TALSVERYFA   80
ICFPLRAKVV VTKGRVKLVI  100

         110        120
          .          .
LVIWAVAFCS AGPIFVLVGV  120
EHDNGTDPRD TNECRATEFA  140
VRSGLLTVMV WVSSVFFFLP  160
VFCLTVLYSL IGRKLWRRKR  180
GEAAVGSSLR DQNHKQTVKM  200

         210        220
          .          .
LAVVVFAFIL CWLPFHVGRY  220
LFSKSLEPGS VEIAQISQYC  240
NLVSFVLFYL SAAINPILYN  260
IMSKKYRVAV FKLLGFEPFS  280
QRKLSTLKDE SSRAWTESSI  300

         310        320
          .          .
NT 302
```

FIG.2

```
1                                                          30
LTLPDLGWDA  PPENDSLVEE  LLPLFPTPLL

HELIX 1                                                    60
AGVTATCVAL  FVVGIAGNLL  TMLVVSRFRE

          HELIX 2                                          90
MRTTTNLYLS  SMAFSDLLIF  ICMPLDLFRL

                                  HELIX 3  120
WQYRPWNLGN  LLCKLFQFVS  ESCTYATVLT

                                                          150
ITALSVERYF  AICFPLRAKV  VVTKGRVKLV

  HELIX 4                                                 180
ILVIWAVAFC  SAGPIFVLVG  VEHDNGTDPR

                                                          210
DTNECRATEF  AVRSGLLTVM  VWVSSVFFFL

HELIX 5                                                   240
PVFCLTVLYS  LIGRKLWRRK  RGEAAVGSSL

                    HELIX 6                               270
RDQNHKQTVK  MLAVVVFAFI  LCWLPFHVGR

                                                          300
YLFSKSLEPG  SVEIAQISQY  CNLVSFVLFY

HELIX 7                                                   330
LSAAINPILY  NIMSKKYRVA  VFKLLGFEPF

                                    353
SQRKLSTLKD  ESSRAWTESS  INT
```

FIG.3

```
          10         20         30         40
          .          .          .          .
GCAGCCTCTCACTTCCCTCTTTCCTCTCCTAGCATCCTCC    40
CTGAGAGCCCGCGCTCGATACTCCTTTGCACTCTTTCGCG    80
CCTAAGAGAACCTTCTCTGGGACCAGCCGGCTCCACCCTC   120
TCGGTCCTATCCAAGAGCCAGTTAAGCAGAGCCCTAAGCA  160
TGTGGAACGCGACCCCGAGCGAGGAACCGGGGCCCAACCT  200

          210        220        230        240
          .          .          .          .
CACGCTGCCAGACCTGGGCTGGGACGCTCCCCCTGAAAAC  240
GACTCGCTAGTGGAGGAGCTGCTGCCGCTCTTCCCCACGC  280
CGCTGTTGGCGGGCGTCACCGCCACCTGCGTGGCGCTCTT  320
CGTGGTGGGTATCGCGGGCAACCTGCTCACGATGCTGGTA  360
GTGTCACGCTTCCGCGAGATGCGCACCACCACCAACCTCT  400

          410        420        430        440
          .          .          .          .
ACCTGTCCAGCATGGCCTTCTCCGACCTACTCATCTTCCT  440
CTGCATGCCCCTCGACCTCTTCCGCCTTTGGCAGTACCGG  480
CCTTGGAACCTTGGCAACCTGCTCTGCAAACTCTTCCAGT  520
TCGTTAGCGAGAGCTGCACCTACGCCACAGTGCTCACCAT  560
CACCGCGCTGAGCGTCGAGCGCTACTTCGCCATCTGCTTC  600

          610        620        630        640
          .          .          .          .
CCGCTGCGGGCCAAGGTAGTGGTCACCAAGGGCCGGGTAA  640
AGCTGGTCATCCTGGTCATCTGGGCCGTGGCCTTCTGCAG  680
CGCCGGGCCCATCTTCGTGCTGGTCGGAGTGGAGCATGAT  720
AACGGCACTGACCCTCGGGACACCAACGAGTGCCGCGCCA  760
CGGAGTTCGCCGTGCGCTCCGGGCTGCTTACCGTCATGGT  800

          810        820        830        840
          .          .          .          .
CTGGGTGTCCAGTGTCTTCTTCTTCCTGCCTGTCTTCTGC  840
CTCACTGTGCTCTATAGCCTCATCGGCAGGAAGCTCTGGC  880
GGAGGAAGCGCGGCGAGGCGGCGGTGGGCTCCTCGCTCAG  920
GGACCAGAACCACAAACAAACCGTGAAAATGCTGGGTGGG  960
TCTCAATGCGCCCTCGAGCTTTCTCTCCCGGGTCCCCTCC 1000

          1010       1020       1030       1040
          .          .          .          .
ACTCCTCGTGCCTTTTCTCTTCTCCCTGA 1029
```

FIG.4

```
          10         20         30         40
          .          .          .          .
MWNATPSEEP GPNLTLPDLG WDAPPENDSL VEELLPLFPT   40
PLLAGVTATC VALFVVGIAG NLLTMLVVSR FREMRTTTNL   80
YLSSMAFSDL LIFLCMPLDL FRLWQYRPWN LGNLLCKLFQ  120
FVSESCTYAT VLTITALSVE RYFAICFPLR AKVVVTKGRV  160
KLVILVIWAV AFCSAGPIFV LVGVEHDNGT DPRDTNECRA  200

          210        220        230        240
          .          .          .          .
TEFAVRSGLL TVMVWVSSVF FFLPVFCLTV LYSLIGRKLW  240
RRKRGEAAVG SSLRDQNHKQ TVKMLGGSQC ALELSLPGPL  280
HSSCLFSSP  289
```

FIG.5

```
          10         20         30         40
          .          .          .          .
CGCCCAGCGAAGAGCCGGGGTTCAACCTCACACTGGCCGA    40
CCTGGACTGGGATGCTTCCCCCGGCAACGACTCGCTGGGC    80
GACGAGCTGCTGCAGCTCTTCCCCGCGCCGCTGCTGGCGG   120
GCGTCACAGCCACCTGCGTGGCACTCTTCGTGGTGGGTAT   160
CGCTGGCAACCTGCTCACCATGCTGGTGGTGTCGCGCTTC   200

          210        220        230        240
          .          .          .          .
CGCGAGCTGCGCACCACCACCAACCTCTACCTGTCCAGCA   240
TGGCCTTCTCCGATCTGCTCATCTTCCTCTGCATGCCCCT   280
GGACCTCGTTCGCCTCTGGCAGTACCGGCCCTGGAACTTC   320
GGCGACCTCCTCTGCAAACTCTTCCAATTCGTCAGTGAGA   360
GCTGCACCTACGCCACGGTGCTCACCATCACAGCGCTGAG   400

          410        420        430        440
          .          .          .          .
CGTCGAGCGCTACTTCGCCATCTGCTTCCCACTCCGGGCC   440
AAGGTGGTGGTCACCAAGGGGCGGGTGAAGCTGGTCATCT   480
TCGTCATCTGGGCCGTGGCCTTCTGCAGCGCCGGGCCCAT   520
CTTCGTGCTAGTCGGGGTGGAGCACGAGAACGGCACCGAC   560
CCTTGGGACACCAACGAGTGCCGCCCCACCGAGTTTGCGG   600

          610        620        630        640
          .          .          .          .
TGCGCTCTGGACTGCTCACGGTCATGGTGTGGGTGTCCAG   640
CATCTTCTTCTTCCTTCCTGTCTTCTGTCTCACGGTCCTC   680
TACAGTCTCATCGGCAGGAAGCTGTGGCGGAGGAGGCGCG   720
GCGATGCTGTCGTGGGTGCCTCGCTCAGGGACCAGAACCA   760
CAAGCAAACCGTGAAAATGCTGGCTGTAGTGGTGTTTGCC   800

          810        820        830        840
          .          .          .          .
TTCATCCTCTGCTGGCTCCCCTTCCACGTAGGGCGATATT   840
TATTTTCCAAATCCTTTGAGCCTGGCTCCTTGGAGATTGC   880
TCAGATCAGCCAGTACTGCAACCTCGTGTCCTTTGTCCTC   920
TTCTACCTCAGTGCTGCCATCAACCCCATTCTGTACAACA   960
TCATGTCCAAGAAGTACCGGGTGGCAGTGTTCAGACTTCT  1000

          1010       1020       1030       1040
          .          .          .          .
GGGATTCGAACCCTTCTCCCAGAGAAAGCTCTCCACTCTG  1040
AAAGATGAAAGTTCTCGGGCCTGGACAGAATCTAGTATTA  1080
ATACATGA  1088
```

FIG.6

```
          10                  20
          .                   .
MLVVSRFREL  RTTTNLYLSS   20
MAFSDLLIFL  CMPLDLVRLW   40
QYRPWNFGDL  LCKLFQFVSE   60
SCTYATVLTI  TALSVERYFA   80
ICFPLRAKVV  VTKGRVKLVI  100

         110                 120
          .                   .
FVIWAVAFCS  AGPIFVLVGV  120
EHENGTDPWD  TNECRPTEFA  140
VRSGLLIVMV  WVSSIIIILP  160
VFCLTVLYSL  IGRKLWRRRR  180
GDAVVGASLR  DQNHKQTVKM  200

         210                 220
          .                   .
LAVVVFAFIL  CWLPFHVGRY  220
LFSKSFEPGS  LEIAQISQYC  240
NLVSFVLFYL  SAAINPILYN  260
IMSKKYRVAV  FRLLGFEPFS  280
QRKLSTLKDE  SSRAWTESSI  300

         310                 320
          .                   .
NT  302
```

FIG.7

```
1                                                  30
PSEEPGFNLT  LADLDWDASP  GNDSLGDELL

                                HELIX 1        60
QLFPAPLLAG  VTATCVALFV  VGIAGNLLTM

                                HELIX 2        90
LVVSRFRELR  TTTNLYLSSM  AFSDLLIFLC

                                                  120
MPLDLVRLWQ  YRPWNFGDLL  CKLFQFVSES

HELIX 3                                           150
CTYATVLTIT  ALSVERYFAI  CFPLRAKVVV

                          HELIX 4                 180
TKGRVKLVIF  VIWAVAFCSA  GPIFVLVGVE

                                                  210
HENGTDPWDT  NECRPTEFAV  RSGLLTVMVW

     HELIX 5                                      240
VSSIFFFLPV  FCLTVLYSLI  GRKLWRRRRG

                                HELIX 6 270
DAVVGASLRD  QNHKQTVKML  AVVVFAFILC

                                                  300
WLPFHVGRYL  FSKSFEPGSL  EIAQISQYCN

          HELIX 7                                 330
LVSFVLFYLS  AAINPILYNI  MSKKYRVAVF

                                                  360
RLLGFEPFSQ  RKLSTLKDES  SRAWTESSIN

361
T
```

FIG.8

```
          10         20         30         40
          .          .          .          .
GCGCCTCACGCTCCCGCTTCGCGGCGCCTGGTCCCTGCGG    40
TCCCCACTCGCTGCGACGCTTTGGGAAGTGCGAGATGGAA    80
CTGGATCGAGAACGCAAATGCGAGGCAGGGCTGGTGACAG   120
CATCCTCCCTACGCGTCTGCACCCGCTCCTCCCTCGCACC   160
CTCCCGCGCCTAAGCGGACCTCCTCGGGAGCCAGCTCGGT   200

         210        220        230        240
          .          .          .          .
CCAGCCTCCCAGCGCAGTCACGTCCCAGAGCCTGTTCAGC   240
TGAGCCGGCAGCATGTGGAACGCGACGCCCAGCGAAGAGC   280
CGGGGTTCAACCTCACACTGGCCGACCTGGACTGGGATGC   320
TTCCCCCGGCAACGACTCGCTGGGCGACGAGCTGCTGCAG   360
CTCTTCCCCGCGCCGCTGCTGGCGGGCGTCACAGCCACCT   400

         410        420        430        440
          .          .          .          .
GCGTGGCACTCTTCGTGGTGGGTATCGCTGGCAACCTGCT   440
CACCATGCTGGTGGTGTCGCGCTTCCGCGAGCTGCGCACC   480
ACCACCAACCTCTACCTGTCCAGCATGGCCTTCTCCGATC   520
TGCTCATCTTCCTCTGCATGCCCCTGGACCTCGTTCGCCT   560
CTGGCAGTACCGGCCCTGGAACTTCGGCGACCTCCTCTGC   600

         610        620        630        640
          .          .          .          .
AAACTCTTCCAATTCGTCAGTGAGAGCTGCACCTACGCCA   640
CGGTGCTCACCATCACAGCGCTGAGCGTCGAGCGCTACTT   680
CGCCATCTGCTTCCCACTCCGGGCCAAGGTGGTGGTCACC   720
AAGGGGCGGGTGAAGCTGGTCATCTTCGTCATCTGGGCCG   760
TGGCCTTCTGCAGCGCCGGGCCCATCTTCGTGCTAGTCGG   800

         810        820        830        840
          .          .          .          .
GGTGGAGCACGAGAACGGCACCGACCCTTGGGACACCAAC   840
GAGTGCCGCCCCACCGAGTTTGCGGTGCGCTCTGGACTGC   880
TCACGGTCATGGTGTGGGTGTCCAGCATCTTCTTCTTCCT   920
TCCTGTCTTCTGTCTCACGGTCCTCTACAGTCTCATCGGC   960
AGGAAGCTGTGGCGGAGGAGGCGCGGCGATGCTGTCGTGG  1000
```

FIG.9A

```
          1010        1020        1030        1040
           .           .           .           .
GTGCCTCGCTCAGGGACCAGAACCACAAGCAAACCGTGAA  1040
AATGCTGGGTGGGTCTCAGCGCGCGCTCAGGCTTTCTCTC  1080
GCGGGTCCTATCCTCTCCCTGTGCCTTCTCCCTTCTCTCT  1120
GA  1122
```

FIG.9B

```
          10         20         30         40
          .          .          .          .
MWNATPSEEPGFNLTLADLDWDASPGNDSLGDELLQLFPA   40
PLLAGVTATCVALFVVGIAGNLLTMLVVSRFRELRTTTNL   80
YLSSMAFSDLLIFLCMPLDLVRLWQYRPWNFGDLLCKLFQ  120
FVSESCTYATVLTITALSVERYFAICFPLRAKVVVTKGRV  160
KLVIFVIWAVAFCSAGPIFVLVGVEHENGTDPWDTNECRP  200

          210        220        230        240
          .          .          .          .
TEFAVRSGLLTVMVWVSSIFFFLPVFCLTVLYSLIGRKLW  240
RRRRGDAVVGASLRDQNHKQTVKMLGGSQRALRLSLAGPI  280
LSLCLLPSL  289
```

FIG.10

```
          10         20         30         40
          .          .          .          .
MPLDLVRLWQYRPWNFGDLLCKLFQFVSESCTYATVLTIT   40
ALSVERYFAICFPLRAKVVVTKGRVKLVIFVIWAVAFCSA   80
GPIFVLVGVEHENGTDPWDTNECRPTEFAVRSGLLTVMVW  120
VSSIFFFLPVFCLTVLYSLIGRKLWRRRRGDAVVGASLRD  160
QNHKQTVKMLAVVVFAFILCWLPFHVGRYLFSKSFEPGSL  200

          210        220        230        240
          .          .          .          .
EIAQISQYCNLVSFVLFYLSAAINPILYNIMSKKYRVAVF  240
RLLGFEPFSQRKLSTLKDESSRAWTESSINT  271
```

FIG.12

```
          10         20         30         40
          .          .          .          .
ATCTGCTCATCTTCCTCTGCATGCCCCTGGACCTCGTTCG    40
CCTCTGGCAGTACCGGCCCTGGAACTTCGGCGACCTCCTC    80
TGCAAACTCTTCCAATTCGTCAGTGAGAGCTGCACCTACG   120
CCACGGTGCTCACCATCACAGCGCTGAGCGTCGAGCGCTA   160
CTTCGCCATCTGCTTCCCACTCCGGGCCAAGGTGGTGGTC   200

          210        220        230        240
          .          .          .          .
ACCAAGGGGCGGGTGAAGCTGGTCATCTTCGTCATCTGGG    240
CCGTGGCCTTCTGCAGCGCCGGGCCCATCTTCGTGCTAGT   280
CGGGGTGGAGCACGAGAACGGCACCGACCCTTGGGACACC   320
AACGAGTGCCGCCCCACCGAGTTTGCGGTGCGCTCTGGAC   360
TGCTCACGGTCATGGTGTGGGTGTCCAGCATCTTCTTCTT   400

          410        420        430        440
          .          .          .          .
CCTTCCTGTCTTCTGTCTCACGGTCCTCTACAGTCTCATC    440
GGCAGGAAGCTGTGGCGGAGGAGGCGCGGCGATGCTGTCG   480
TGGGTGCCTCGCTCAGGGACCAGAACCACAAGCAAACCGT   520
GAAAATGCTGGCTGTAGTGGTGTTTGCCTTCATCCTCTGC   560
TGGCTCCCCTTCCACGTAGGGCGATATTTATTTTCCAAAT   600

          610        620        630        640
          .          .          .          .
CCTTTGAGCCTGGCTCCTTGGAGATTGCTCAGATCAGCCA    640
GTACTGCAACCTCGTGTCCTTTGTCCTCTTCTACCTCAGT   680
GCTGCCATCAACCCCATTCTGTACAACATCATGTCCAAGA   720
AGTACCGGGTGGCAGTGTTCAGACTTCTGGGATTCGAACC   760
CTTCTCCCAGAGAAAGCTCTCCACTCTGAAAGATGAAAGT   800

          810        820        830        840
          .          .          .          .
TCTCGGGCCTGGACAGAATCTAGTATTAATACATGA      836
```

FIG.11

```
                                        v10         v20
FIG.3-SWINE TYPE I CLONE 7-3orf     LTLPDLGWDAPPENDSLVEE
                                    LTLPDLGWDAPPENDSLVEE
FIG.5-SWINE TYPE II CLONE 1375m     LTLPDLGWDAPPENDSLVEE
                                         ^20         ^30
                                           v30         v40
FIG.3-SWINE TYPE I CLONE 7-3orf     LLPLFPTPLLAGVTATCVAL
                                    LLPLFPTPLLAGVTATCVAL
FIG.5-SWINE TYPE II CLONE 1375m     LLPLFPTPLLAGVTATCVAL
                                         ^40         ^50
                                           v50         v60
FIG.3-SWINE TYPE I CLONE 7-3orf     FVVGIAGNLLTMLVVSRFRE
                                    FVVGIAGNLLTMLVVSRFRE
FIG.5-SWINE TYPE II CLONE 1375m     FVVGIAGNLLTMLVVSRFRE
                                         ^60         ^70
                                           v70         v80
FIG.3-SWINE TYPE I CLONE 7-3orf     MRTTTNLYLSSMAFSDLLIF
                                    MRTTTNLYLSSMAFSDLLIF
FIG.5-SWINE TYPE II CLONE 1375m     MRTTTNLYLSSMAFSDLLIF
                                         ^80         ^90
                                           v90         v100
FIG.3-SWINE TYPE I CLONE 7-3orf     LCMPLDLFRLWQYRPWNLGN
                                    LCMPLDLFRLWQYRPWNLGN
FIG.5-SWINE TYPE II CLONE 1375m     LCMPLDLFRLWQYRPWNLGN
                                         ^100        ^110
                                           v110        v120
FIG.3-SWINE TYPE I CLONE 7-3orf     LLCKLFQFVSESCTYATVLT
                                    LLCKLFQFVSESCTYATVLT
FIG.5-SWINE TYPE II CLONE 1375m     LLCKLFQFVSESCTYATVLT
                                         ^120        ^130
                                           v130        v140
FIG.3-SWINE TYPE I CLONE 7-3orf     ITALSVERYFAICFPLRAKV
                                    ITALSVERYFAICFPLRAKV
FIG.5-SWINE TYPE II CLONE 1375m     ITALSVERYFAICFPLRAKV
                                         ^140        ^150
                                           v150        v160
FIG.3-SWINE TYPE I CLONE 7-3orf     VVTKGRVKLVILVIWAVAFC
                                    VVTKGRVKLVILVIWAVAFC
FIG.5-SWINE TYPE II CLONE 1375m     VVTKGRVKLVILVIWAVAFC
                                         ^160        ^170
```

FIG.13A

```
                                        v170        v180
FIG.3-SWINE TYPE I CLONE 7-3orf     SAGPIFVLVGVEHDNGTDPR
                                    SAGPIFVLVGVEHDNGTDPR
FIG.5-SWINE TYPE II CLONE 1375m     SAGPIFVLVGVEHDNGTDPR
                                         ^180        ^190
                                        v190        v200
FIG.3-SWINE TYPE I CLONE 7-3orf     DTNECRATEFAVRSGLLTVM
                                    DTNECRATEFAVRSGLLTVM
FIG.5-SWINE TYPE II CLONE 1375m     DTNECRATEFAVRSGLLTVM
                                         ^200        ^210
                                        v210        v220
FIG.3-SWINE TYPE I CLONE 7-3orf     VWVSSVFFFLPVFCLTVLYS
                                    VWVSSVFFFLPVFCLTVLYS
FIG.5-SWINE TYPE II CLONE 1375m     VWVSSVFFFLPVFCLTVLYS
                                         ^220        ^230
                                        v230        v240
FIG.3-SWINE TYPE I CLONE 7-3orf     LIGRKLWRRKRGEAAVGSSL
                                    LIGRKLWRRKRGEAAVGSSL
FIG.5-SWINE TYPE II CLONE 1375m     LIGRKLWRRKRGEAAVGSSL
                                         ^240        ^250
                                        v250        v260
FIG.3-SWINE TYPE I CLONE 7-3orf     RDQNHKQTVKMLAVVVFAFI
                                    RDQNHKQTVKML:     A:
FIG.5-SWINE TYPE II CLONE 1375m     RDQNHKQTVKMLGGSQCALE
                                         ^260        ^270
                                        v270
FIG.3-SWINE TYPE I CLONE 7-3orf     LCWL-PFHVGRYLFSKS
                                    L.   P:H :..LFS.:
FIG.5-SWINE TYPE II CLONE 1375m     LSLPGPLH-SSCLFSSP
                                         ^280
```

FIG.13B

```
                                        v10        v20
FIG.8-HUMAN TYPE I 1146orf              PSEEPGFNLTLADLDWDASP
                                        PSEEPGFNLTLADLDWDASP
FIG.10-HUMAN TYPE II CLONE1141m         PSEEPGFNLTLADLDWDASP
                                            ^10        ^20
                                                v30        v40
FIG.8-HUMAN TYPE I 1146orf              GNDSLGDELLQLFPAPLLAG
                                        GNDSLGDELLQLFPAPLLAG
FIG.10-HUMAN TYPE II CLONE1141m         GNDSLGDELLQLFPAPLLAG
                                            ^30        ^40
                                                v50        v60
FIG.8-HUMAN TYPE I 1146orf              VTATCVALFVVGIAGNLLTM
                                        VTATCVALFVVGIAGNLLTM
FIG.10-HUMAN TYPE II CLONE1141m         VTATCVALFVVGIAGNLLTM
                                            ^50        ^60
                                                v70        v80
FIG.8-HUMAN TYPE I 1146orf              LVVSRFRELRTTTNLYLSSM
                                        LVVSRFRELRTTTNLYLSSM
FIG.10-HUMAN TYPE II CLONE1141m         LVVSRFRELRTTTNLYLSSM
                                            ^70        ^80
                                                v90        v100
FIG.8-HUMAN TYPE I 1146orf              AFSDLLIFLCMPLDLVRLWQ
                                        AFSDLLIFLCMPLDLVRLWQ
FIG.10-HUMAN TYPE II CLONE1141m         AFSDLLIFLCMPLDLVRLWQ
                                            ^90        ^100
                                                v110       v120
FIG.8-HUMAN TYPE I 1146orf              YRPWNFGDLLCKLFQFVSES
                                        YRPWNFGDLLCKLFQFVSES
FIG.10-HUMAN TYPE II CLONE1141m         YRPWNFGDLLCKLFQFVSES
                                            ^100       ^110
                                                v130       v140
FIG.8-HUMAN TYPE I 1146orf              CTYATVLTITALSVERYFAI
                                        CTYATVLTITALSVERYFAI
FIG.10-HUMAN TYPE II CLONE1141m         CTYATVLTITALSVERYFAI
                                            ^130       ^140

                                                v150       v160
FIG.8-HUMAN TYPE I 1146orf              CFPLRAKVVVTKGRVKLVIF
                                        CFPLRAKVVVTKGRVKLVIF
FIG.10-HUMAN TYPE II CLONE1141m         CFPLRAKVVVTKGRVKLVIF
                                            ^150       ^160
```

FIG.14A

```
                                        v170      v180
FIG.8-HUMAN TYPE I 1146orf              VIWAVAFCSAGPIFVLVGVE
                                        VIWAVAFCSAGPIFVLVGVE
FIG.10-HUMAN TYPE II CLONE1141m         VIWAVAFCSAGPIFVLVGVE
                                         ^170      ^180
                                            v190      v200
FIG.8-HUMAN TYPE I 1146orf              HENGTDPWDTNECRPTEFAV
                                        HENGTDPWDTNECRPTEFAV
FIG.10-HUMAN TYPE II CLONE1141m         HENGTDPWDTNECRPTEFAV
                                         ^190      ^200
                                            v210      v220
FIG.8-HUMAN TYPE I 1146orf              RSGLLTVMVWVSSIFFFLPV
                                        RSGLLTVMVWVSSIFFFLPV
FIG.10-HUMAN TYPE II CLONE1141m         RSGLLTVMVWVSSIFFFLPV
                                         ^210      ^220
                                            v230      v240
FIG.8-HUMAN TYPE I 1146orf              FCLTVLYSLIGRKLWRRRRG
                                        FCLTVLYSLIGRKLWRRRRG
FIG.10-HUMAN TYPE II CLONE1141m         FCLTVLYSLIGRKLWRRRRG
                                         ^230      ^240
                                            v250      v260
FIG.8-HUMAN TYPE I 1146orf              DAVVGASLRDQNHKQTVKML
                                        DAVVGASLRDQNHKQTVKML
FIG.10-HUMAN TYPE II CLONE1141m         DAVVGASLRDQNHKQTVKML
                                         ^250      ^260
```

FIG.14B

```
                    v10        v20        v30        v40
FIG.3-SWINE TYPE I CLONE 7-3orf  LTLPDLGWDAPPENDSLVEELLPLFPTPLLAGVTATCVAL
                                 LTL:DL:WDA:P.NDSL :ELL.LFP:PLLAGVTATCVAL
FIG.8-HUMAN TYPE I 1146orf       LTLADLDWDASPGNDSLGDELLQLFPAPLLAGVTATCVAL
                                  ^10        ^20        ^30        ^40
                    v50        v60        v70        v80
FIG.3-SWINE TYPE I CLONE 7-3orf  FVVGIAGNLLTMLVVSRFREMRTTTNLYLSSMAFSDLLIF
                                 FVVGIAGNLLTMLVVSRFRE:RTTTNLYLSSMAFSDLLIF
FIG.8-HUMAN TYPE I 1146orf       FVVGIAGNLLTMLVVSRFRELRTTTNLYLSSMAFSDLLIF
                                  ^50        ^60        ^70        ^80
                    v90        v100       v110       v120
FIG.3-SWINE TYPE I CLONE 7-3orf  LCMPLDLFRLWQYRPWNLGNLLCKLFQFVSESCTYATVLT
                                 LCMPLDL RLWQYRPWN:G:LLCKLFQFVSESCTYATVLT
FIG.8-HUMAN TYPE I 1146orf       LCMPLDLVRLWQYRPWNFGDLLCKLFQFVSESCTYATVLT
                                  ^90        ^100       ^110       ^120
                    v130       v140       v150       v160
FIG.3-SWINE TYPE I CLONE 7-3orf  ITALSVERYFAICFPLRAKVVVTKGRVKLVILVIWAVAFC
                                 ITALSVERYFAICFPLRAKVVVTKGRVKLVI:VIWAVAFC
FIG.8-HUMAN TYPE I 1146orf       ITALSVERYFAICFPLRAKVVVTKGRVKLVIFVIWAVAFC
                                  ^130       ^140       ^150       ^160
                    v170       v180       v190       v200
FIG.3-SWINE TYPE I CLONE 7-3orf  SAGPIFVLVGVEHDNGTDPRDTNECRATEFAVRSGLLTVM
                                 SAGPIFVLVGVEH:NGTDP:DTNECR:TEFAVRSGLLTVM
FIG.8-HUMAN TYPE I 1146orf       SAGPIFVLVGVEHENGTDPWDTNECRPTEFAVRSGLLTVM
                                  ^170       ^180       ^190       ^200
                    v210       v220       v230       v240
FIG.3-SWINE TYPE I CLONE 7-3orf  VWVSSVFFFLPVFCLTVLYSLIGRKLWRRKRGEAAVGSSL
                                 VWVSS:FFFLPVFVLTVLYSLIGRKLWRR:RG:A.VG:SL
FIG.8-HUMAN TYPE I 1146orf       VWVSSIFFFLPVFCLTVLYSLIGRKLWRRRRGDAVVGASL
                                  ^210       ^220       ^230       ^240
                    v250       v260       v270       v280
FIG.3-SWINE TYPE I CLONE 7-3orf  RDQNHKQTVKMLAVVVFAFILCWLPFHVGRYLFSKSLEPG
                                 RDQNHKQTVKMLAVVVFAFILCWLPFHVGRYLFSKS:EPG
FIG.8-HUMAN TYPE I 1146orf       RDQNHKQTVKMLAVVVFAFILCWLPFHVGRYLFSKSFEPG
                                  ^250       ^260       ^270       ^280
                    v290       v300       v310       v320
FIG.3-SWINE TYPE I CLONE 7-3orf  SVEIAQISQYCNLVSFVLFYLSAAINPILYNIMSKKYRVA
                                 S:EIAQISQYCNLVSFVLFYLSAAINPILYNIMSKKYRVA
FIG.8-HUMAN TYPE I 1146orf       SLEIAQISQYCNLVSFVLFYLSAAINPILYNIMSKKYRVA
                                  ^290       ^300       ^310       ^320
                    v330       v340       v350
FIG.3-SWINE TYPE I CLONE 7-3orf  VFKLLGFEPFSQRKLSTLKDESSRAWTESSINT
                                 VF:LLGFEPFSQRKLSTLKDESSRAWTESSINT
FIG.8-HUMAN TYPE I 1146orf       VFRLLGFEPFSQRKLSTLKDESSRAWTESSINT
                                  ^330       ^340       ^350       ^360
```

FIG.15

```
                                  v10        v20
FIG.5-SWINE TYPE II CLONE 1375m   MWNATPSEEPGPNLTLPDLG
                                  MWNATPSEEPG NLTL:DL:
FIG.10-HUMAN TYPE II CLONE1141m   MWNATPSEEPGFNLTLADLD
                                        ^10        ^20
                                        v30        v40
FIG.5-SWINE TYPE II CLONE 1375m   WDAPPENDSLVEELLPLFPT
                                  WDA:P.NDSL :ELL.LFP:
FIG.10-HUMAN TYPE II CLONE1141m   WDASPGNDSLGDELLQLFPA
                                        ^30        ^40
                                        v50        v60
FIG.5-SWINE TYPE II CLONE 1375m   PLLAGVTATCVALFVVGIAG
                                  PLLAGVTATCVALFVVGIAG
FIG.10-HUMAN TYPE II CLONE1141m   PLLAGVTATCVALFVVGIAG
                                        ^50        ^60
                                        v70        v80
FIG.5-SWINE TYPE II CLONE 1375m   NLLTMLVVSRFREMRTTTNL
                                  NLLTMLVVSRFRE:RTTTNL
FIG.10-HUMAN TYPE II CLONE1141m   NLLTMLVVSRFRELRTTTNL
                                        ^70        ^80
                                        v90        v100
FIG.5-SWINE TYPE II CLONE 1375m   YLSSMAFSDLLIFLCMPLDL
                                  YLSSMAFSDLLIFLCMPLDL
FIG.10-HUMAN TYPE II CLONE1141m   YLSSMAFSDLLIFLCMPLDL
                                        ^90        ^100
                                        v110       v120
FIG.5-SWINE TYPE II CLONE 1375m   FRLWQYRPWNLGNLLCKLFQ
                                   RLWQYRPWN:G:LLCKLFQ
FIG.10-HUMAN TYPE II CLONE1141m   VRLWQYRPWNFGDLLCKLFQ
                                        ^110       ^120
                                        v130       v140
FIG.5-SWINE TYPE II CLONE 1375m   FVSESCTYATVLTITALSVE
                                  FVSESCTYATVLTITALSVE
FIG.10-HUMAN TYPE II CLONE1141m   FVSESCTYATVLTITALSVE
                                        ^130       ^140
                                        v150       v160
FIG.5-SWINE TYPE II CLONE 1375m   RYFAICFPLRAKVVVTKGRV
                                  RYFAICFPLRAKVVVTKGRV
FIG.10-HUMAN TYPE II CLONE1141m   RYFAICFPLRAKVVVTKGRV
                                        ^150       ^160
```

FIG.16A

```
                                    v170        v180
FIG.5-SWINE TYPE II CLONE 1375m     KLVILVIWAVAFCSAGPIFV
                                    KLVI:VIWAVAFCSAGPIFV
FIG.10-HUMAN TYPE II CLONE1141m     KLVIFVIWAVAFCSAGPIFV
                                    ^170        ^180
                                    v190        v200
FIG.5-SWINE TYPE II CLONE 1375m     LVGVEHDNGTDPRDTNECRA
                                    LVGVEH:NGTDP:DTNECR:
FIG.10-HUMAN TYPE II CLONE1141m     LVGVEHENGTDPWDTNECRP
                                    ^190        ^200
                                    v210        v220
FIG.5-SWINE TYPE II CLONE 1375m     TEFAVRSGLLTVMVWVSSVI
                                    TEFAVRSGLLTVMVWVSS:F
FIG.10-HUMAN TYPE II CLONE1141m     TEFAVRSGLLTVMVWVSSIF
                                    ^210        ^220
                                    v230        v240
FIG.5-SWINE TYPE II CLONE 1375m     FFLPVFCLTVLYSLIGRKLW
                                    FFLPVFCLTVLYSLIGRKLW
FIG.10-HUMAN TYPE II CLONE1141m     FFLPVFCLTVLYSLIGRKLW
                                    ^230        ^240
                                    v250        v260
FIG.5-SWINE TYPE II CLONE 1375m     RRKRGEAAVGSSLRDQNHKQ
                                    RR:RG:A.VG:SLRDQNHKQ
FIG.10-HUMAN TYPE II CLONE1141m     RRRRGDAVVGASLRDQNHKQ
                                    ^250        ^260
                                    v270        v280
FIG.5-SWINE TYPE II CLONE 1375m     TVKMLGGSQCALELSLPGPL
                                    TVKMLGGSQ AL LSL:GP:
FIG.10-HUMAN TYPE II CLONE1141m     TVKMLGGSQRALRLSLAGPI
                                    ^270        ^280

FIG.5-SWINE TYPE II CLONE 1375m     HSSCLFSS
                                     S CL::S
FIG.10-HUMAN TYPE II CLONE1141m     LSLCLLPS
```

FIG.16B

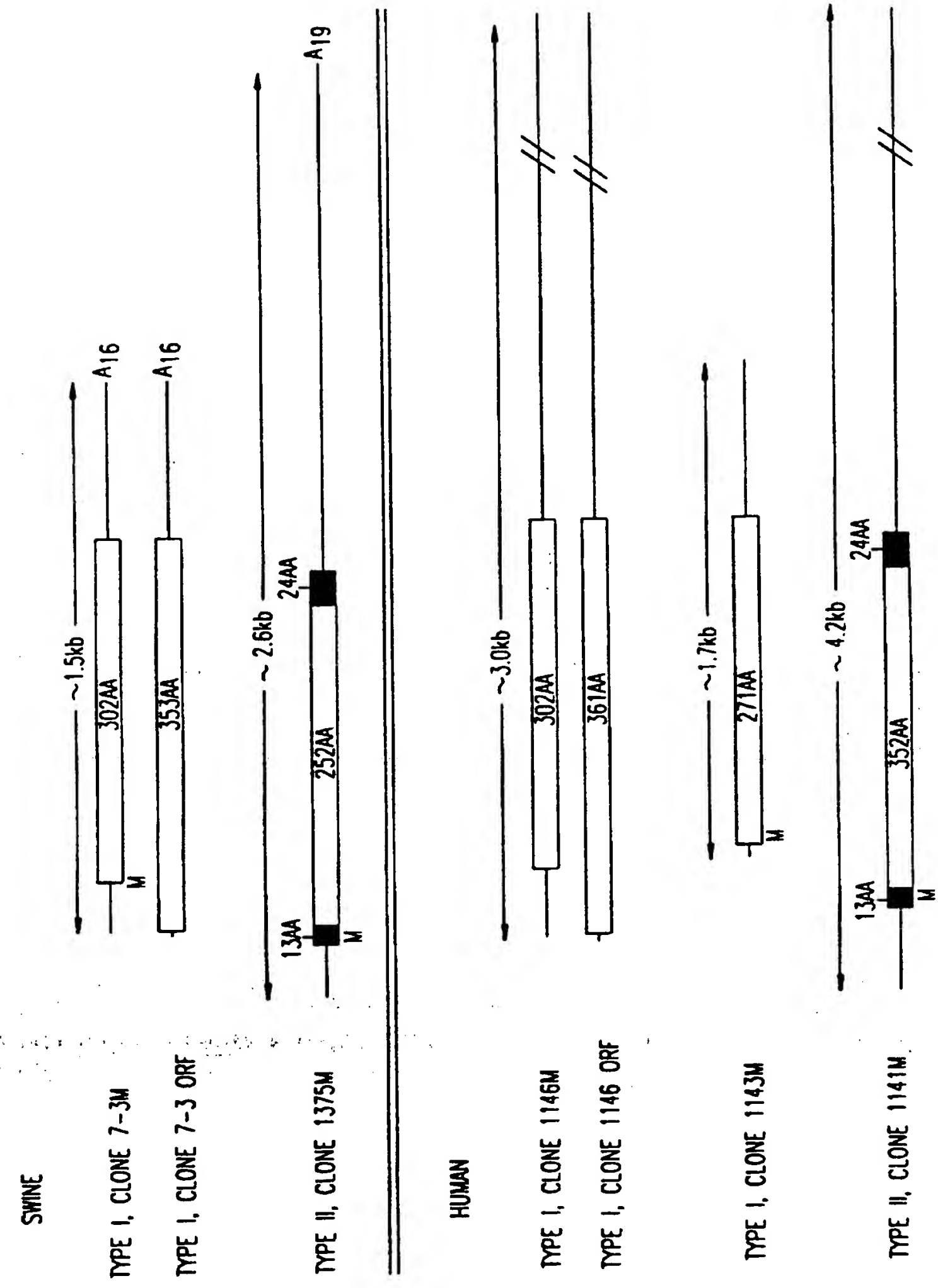
SWINE
TYPE I, CLONE 7-3M
~1.5kb
302AA
M
A16
TYPE I, CLONE 7-3 ORF
353AA
A16
TYPE II, CLONE 1375M
~2.6kb
24AA
13AA
252AA
M
A19
HUMAN
TYPE I, CLONE 1146M
~3.0kb
302AA
TYPE I, CLONE 1146 ORF
361AA
TYPE I, CLONE 1143M
~1.7kb
271AA
M
TYPE II, CLONE 1141M
~4.2kb
24AA
13AA
352AA
M

FIG. 17

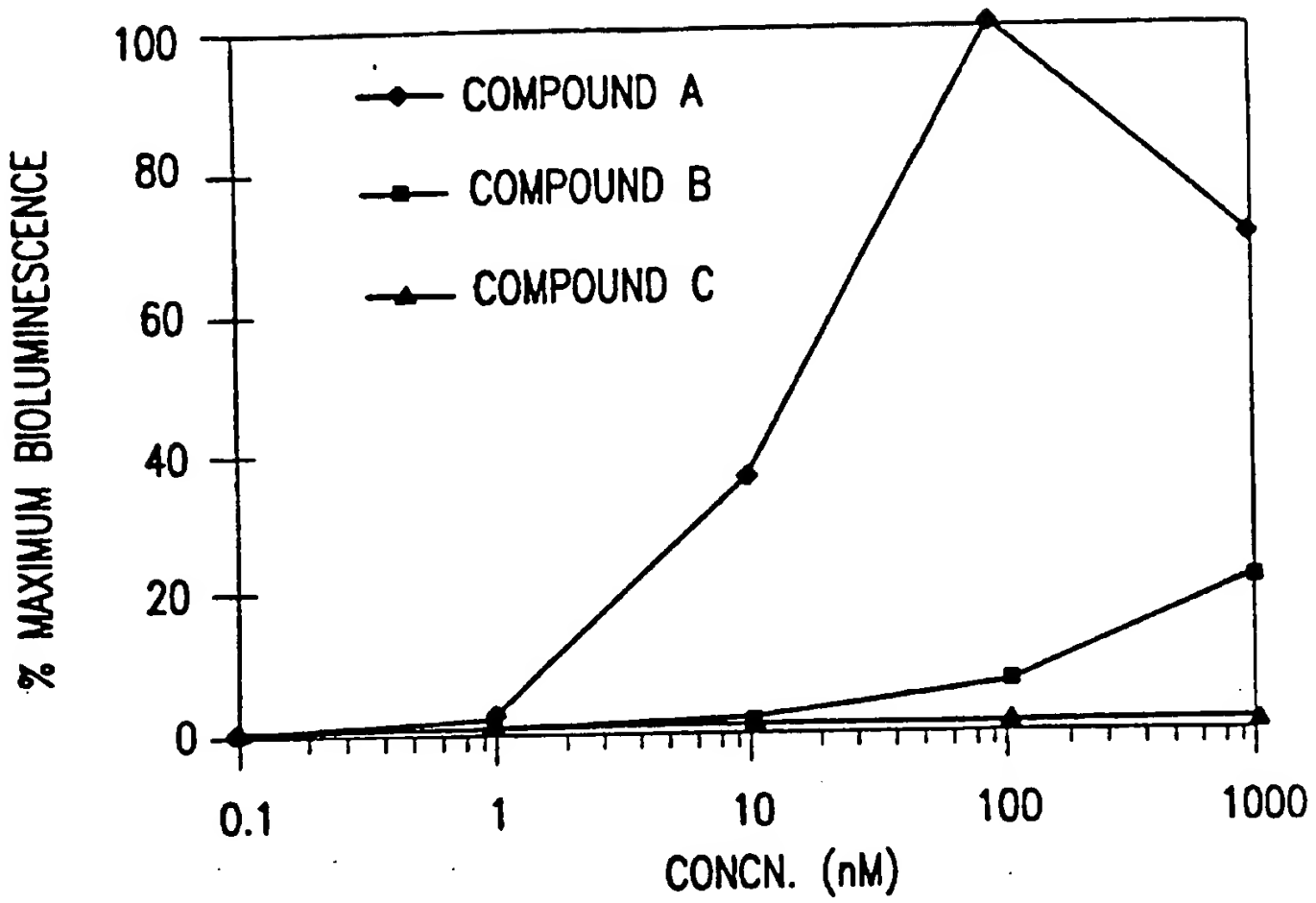

FIG.18

(100Nm)	SWINE CLONE 7-3		HUMAN CLONE 1146	
	24 HOURS	48 HOURS	24 HOURS	48 HOURS
COMPOUND A (100nm) (1000 nM)	13,553 9,176	2,692	1,353 3,091	2,228
COMPOUND B (100nM) COMPOUND C (100nM)	717 100	425 58	113 96	108 67
GHRP-2 (1000 nM) GHRP-6 (1000 nM)	2,492 5,003		1542 617	

FIG.19

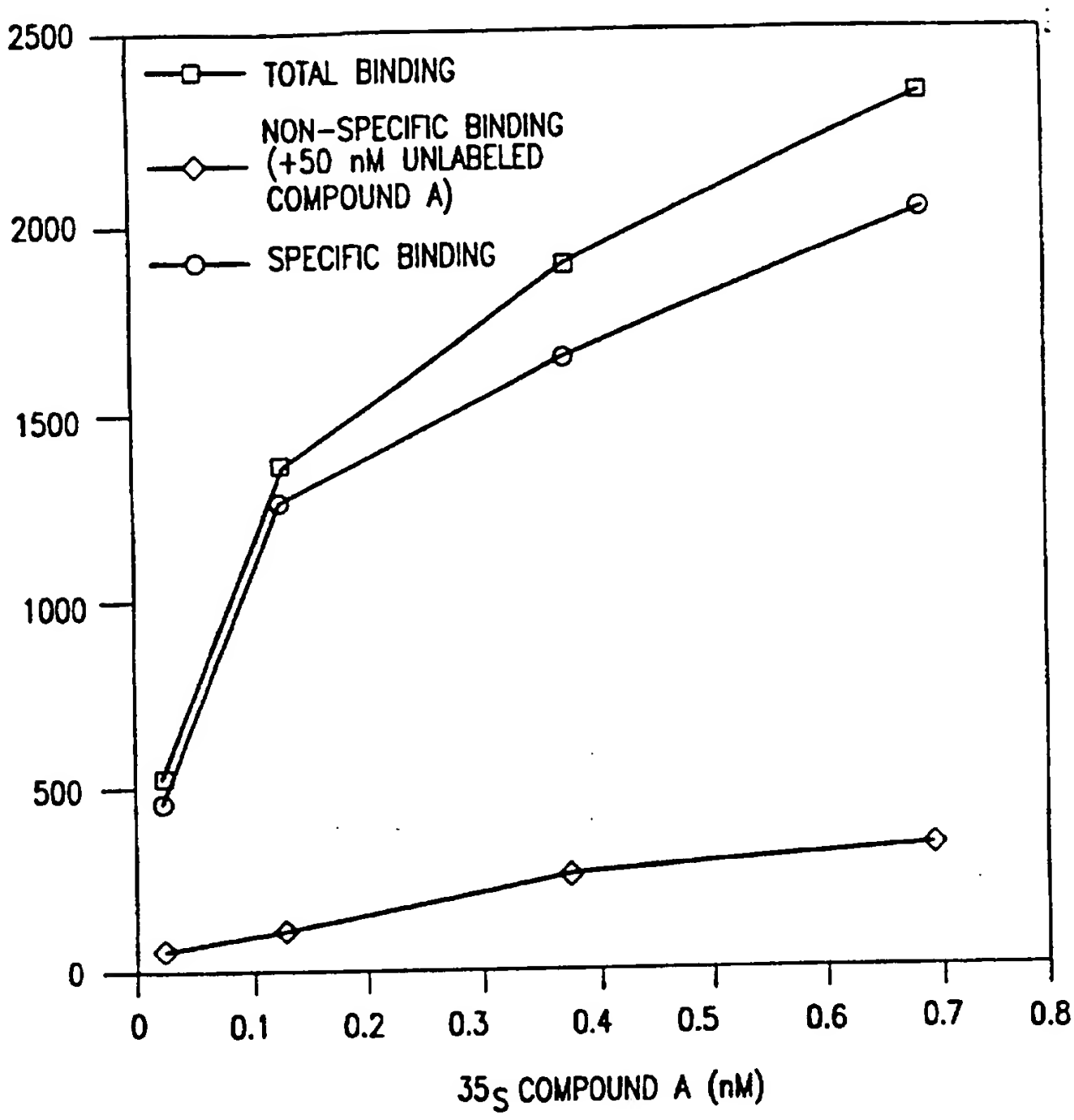

FIG.20

LIGAND	INHIBITION (% OF CONTROL SPECIFIC BINDING)
COMPOUND A @ 5nM	97
GHRP-6 @ 10nM	84
COMPOUND C	
1.692,428 @ 1 μM	43
GALAMIN @ 10 μM	44
AMENOMEDIN N @ 10 μM	19

FIG.21

```
  1  MWNATPSEEP GFNLTLADLD WDASPGNDSL GDELLQLFPA PLLAGVTATC
 51  VALFVVGIAG NLLTMLVVSR FRELRTTTNL YLSSMAFSDL LIFLCMPLDL
101  VRLWQYRPWN FGDLLCKLFQ FVSESCTYAT VLTITALSVE RYFAICFPLR
151  AKVVVTKGRV KLVIFVIWAV AFCSAGPIFV LVGVEHENGT DPWDTNECRP
201  TEFAVRSGLL TVMVWVSSIF FFLPVFCLTV LYSLIGRKLW RRRRGDAVVG
251  ASLRDQNHKQ TVKMLAVVVF AFILCWLPFH VGRYLFSKSF EPGSLEIAQI
301  SQYCNLVSFV LFYLSAAINP ILYNIMSKKY RVAVFRLLGF EPFSQRKLST
351  LKDESSRAWT ESSINT*
```

FIG.22

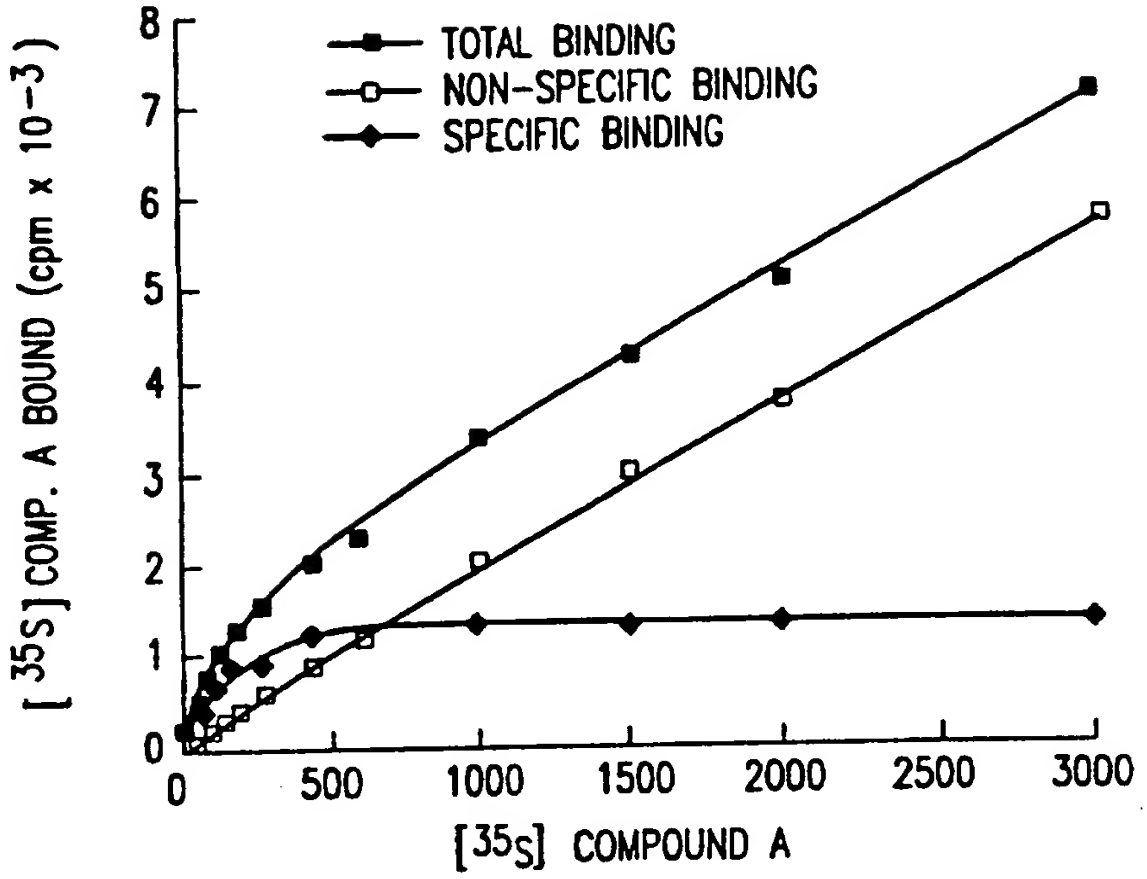

FIG.23A

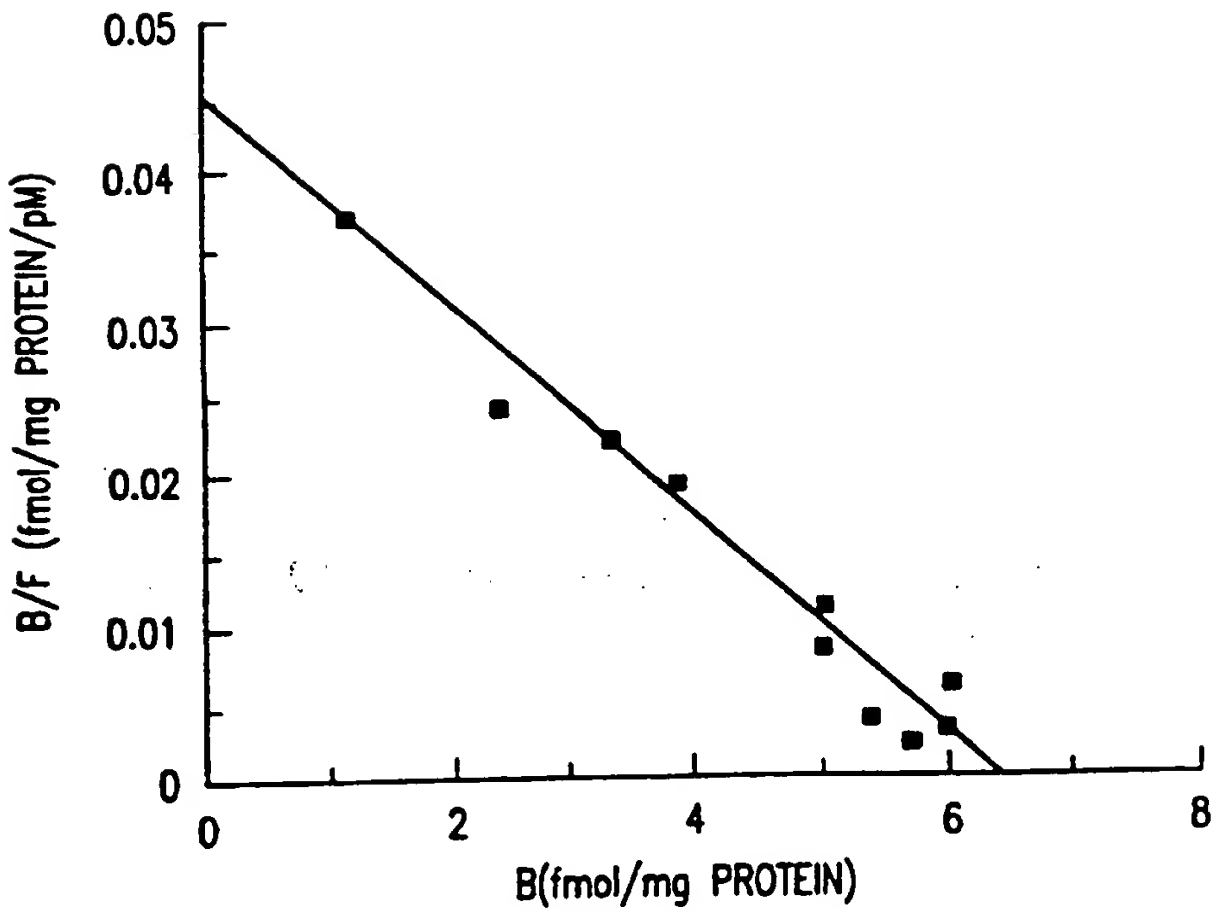

FIG.23B

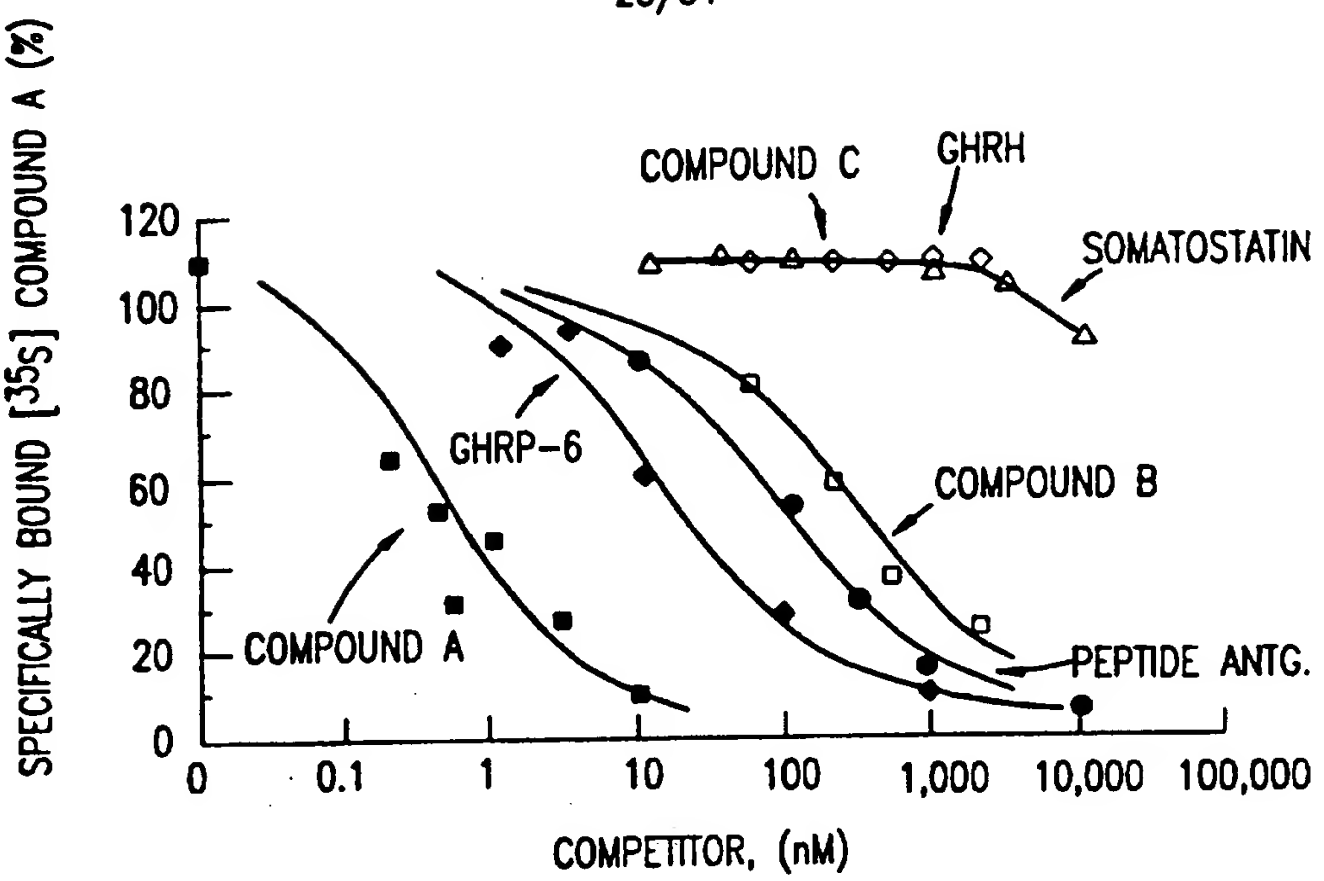
SPECIFICALLY BOUND [35S] COMPOUND A (%)
120
100
80
60
40
20
0
0
0.1
1
10
100
1,000
10,000
100,000
COMPETITOR, (nM)
COMPOUND C
GHRH
SOMATOSTATIN
GHRP-6
COMPOUND B
COMPOUND A
PEPTIDE ANTG.

FIG.24

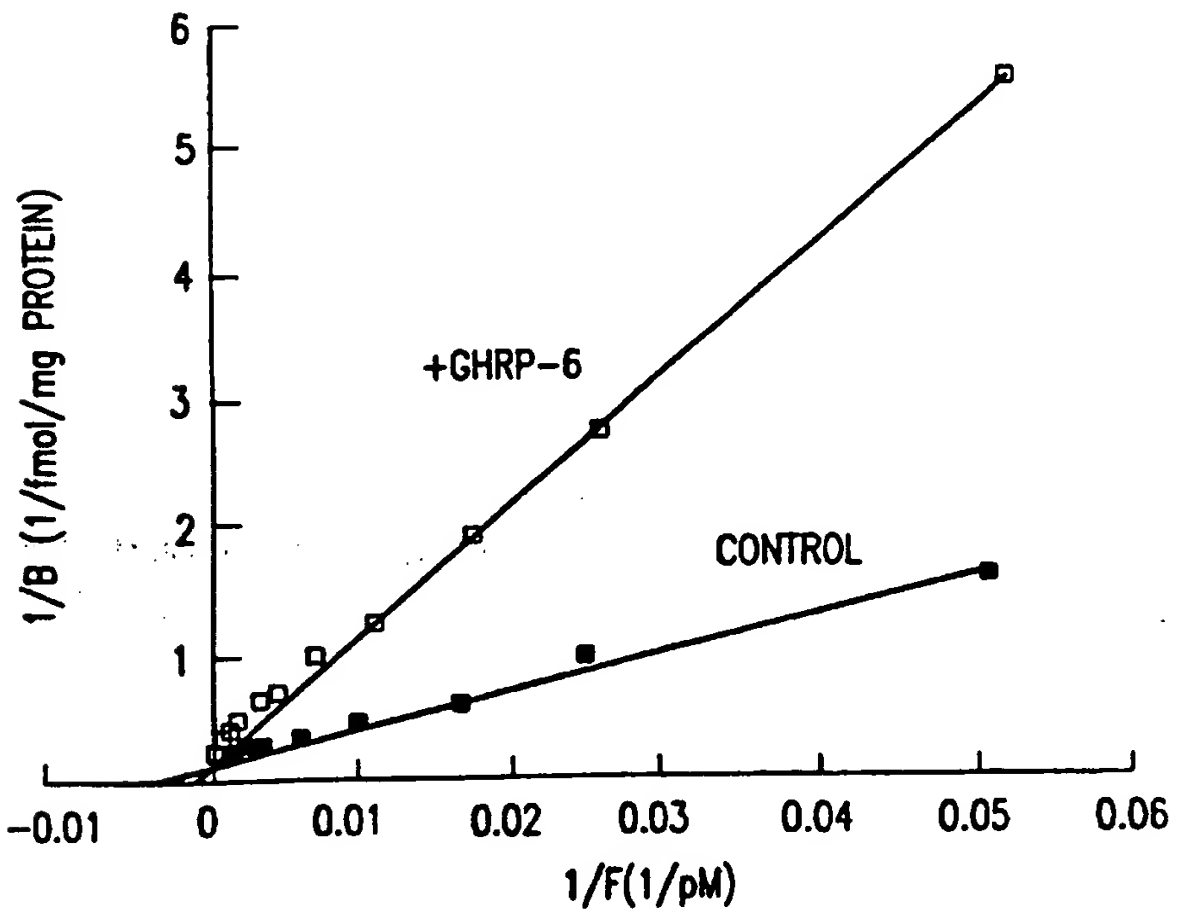
1/B (1/fmol/mg PROTEIN)
6
5
4
3
2
1
+GHRP-6
CONTROL
-0.01
0
0.01
0.02
0.03
0.04
0.05
0.06
1/F(1/pM)

FIG.25

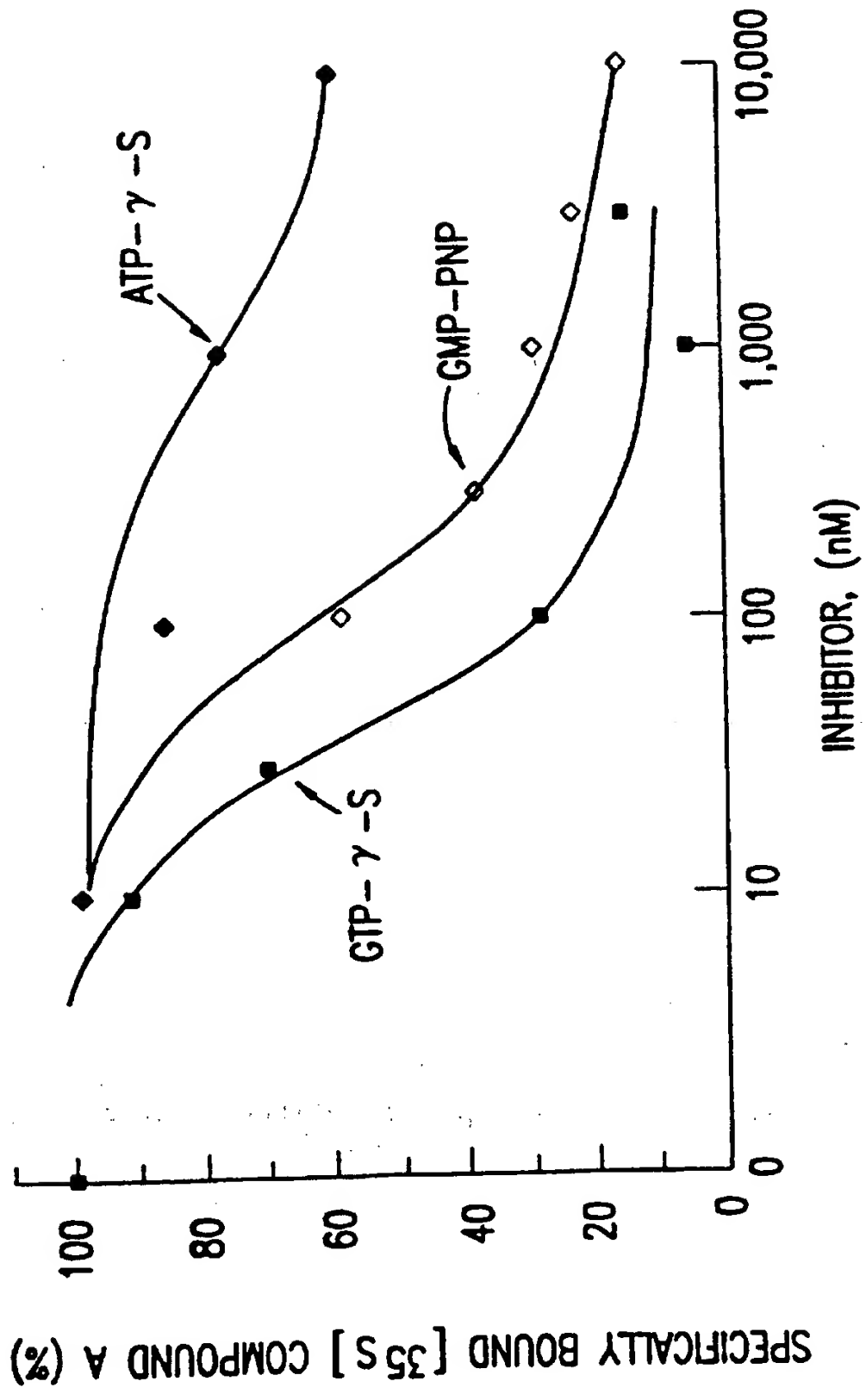
ATP-γ-S
GMP-PNP
GTP-γ-S
10,000
1,000
100
10
0
INHIBITOR, (nM)
100
80
60
40
20
0
SPECIFICALLY BOUND [35S] COMPOUND A (%)

FIG.26

```
          10          20          30          40          50          60
ATG TGG AAC GCG ACC CCC AGC GAG GAG CCG GAG CCT AAC GTC ACG TTG GAC CTG GAT TGG   60
GAC GCT TCC CCC GGC AAC GAC TCA CTG CCT GAC GAA CTG CTG CCG CTG TTC CCC GCT CCG  120
CTG CTG GCA GGC GTC ACC GCC ACC TGC GTG GCG CTC TTC GTG GTG GGC ATC TCA GGC AAC  180
CTG CTC ACT ATG CTG GTG GTG TCC CGC TTC CGC GAG CTG CGC ACC ACC ACC AAC CTC TAC  240
CTG TCC AGC ATG GCC TTC TCG GAT CTG CTC ATC TTC CTG TGC ATG CCG CTG GAC CTC GTC  300

          310         320         330         340         350         360
CGC CTC TGG CAG TAC CGG CCC TGG AAC TTC GGC GAC CTG CTC TGC AAA CTC TTC CAG TTT  360
GTC AGC GAG AGC TGC ACC TAC GCC ACG GTC CTC ACC ATC ACC GCG CTG AGC GTC GAG CGC  420
TAC TTC GCC ATC TGC TTC CCT CTG CGG GCC AAG GTG GTG GTC ACT AAG GGC CGC GTG AAG  480
CTG GTC ATC CTT GTC ATC TGG GCC GTG GCT TTC TGC AGC GCG GGG CCC ATC TTC GTG CTG  540
GTG GGC GTG GAG CAC GAA AAC GGC ACA GAT CCC CGG GAC ACC AAC GAA TGC CGC GCC ACC  600

          610         620         630         640         650         660
GAG TTC GCT GTG CGC TCT GGG CTG CTC ACC GTC ATG GTG TGG GTG TCC AGC GTC TTC TTC  660
TTT CTA CCG GTC TTC TGC CTC ACT GTG CTC TAC AGT CTC ATC GGG AGG AAG CTA TCG CGG  720
AGA CGC GGA GAT GCA GCG GTG GGC GCC TCG CTC CGG GAC CAG AAC CAC AAG CAG ACA GTG  780
AAG ATG CTT Ggt gag tcc tgg cac ccg ctg acc ttt ctt ccc cca ctg cct gcc ctt ccc  840
cag cgg cct cta ttt ctg ttt ctc atc atc tcc gct ccc caa gtc tct caa gtc tct gtc  900
```

FIG.27A

910 920 930 940 950 960

```
ttt ctc tgc ctc tct cac ctt ggt tct cgg tct cac tgc ttt ctg ttt tct tcc tgt ctt   960
ttc ctg tat ctt gtc cac gaa aaa gaa ccc tca tat tgg taa ttc ctt aaa acg agg aac  1020
ctt ggt ctg gga aaa ttg gtc caa gat gga aat acc tca cgg ttt att gag ccc cta att  1080
gtt aac ggt tta gct tct tgt ctc aca tag aat ttg tgg tta tca aag taa taa tat taa  1140
ggt aag cag gca ggt aat ggg ttt aga aat cac tcc atg gta agt cta acc aca aat ttg  1200
```

1210 1220 1230 1240 1250 1260

```
ggt cac tct gtt aag gac ggc tta tag atg tat ttt gtt tgt ttt caa tat tgg gat ttg  1260
ttt tct gcc ctg cat ctt tct cag ata att aca tcc act ctg ttt agt cta tgg ttt tgc  1320
cag gag ggg ctt cat gct ggg gtc tcc ttt ttc ttg ttt ttg tat ttg tct ccc cag taa  1380
tat agg cca gga tag ggt gga gaa gtc atc ctt tcc tca aac tgt cct tca gga agg tct  1440
ggg tac tga acg gtt act gca taa act ctg ctt ccc caa agg cat gtg ctt ggt gtg gta  1500
```

1510 1520 1530 1540 1550 1560

```
aag tca tga aga tgg tgc tca tgt cca aga gga acc tct gat ctc act ttt caa ggg att  1560
tca tgt ttg ctg aca ttt aat act tgt tag ttt ttg cag ggg gat gat ttc tca ttt gca  1620
att tta tta ttc tca aat tct gca tgt cag aat gtt aga gat ttc tca ggg atg tca ggt  1680
tct gtt tcc aga tga gtg att gcc ctg tgt cct cca ttg gac tgt aaa ctc ata tgc acc  1740
aga cag ggt cta cat tgc tgc cgt ggt gca tag cct tcc atg tgt cac tta gtc cta aag  1800
```

FIG.27B

1810 1820 1830 1840 1850 1860

aga agt tac taa taa cct aat ctc act aat ctc act ggc atc tca atg ccg atc cca ttg 1860
tca tct gaa aat ttg aag ggg aca tta aag tgg cac agg gac cag aac aat att ttt ctc 1920
tca ttg ctg aat ttt aaa aac aat cta aaa aat tgg aat tct tga aga aac tat ctt ata 1980
tga cta aaa tga agc ctt ggg tgg gtg cta att att att gtc tgg ctt acc tgc ccc ccc 2040
cac tac tta tat ctt tta gag atg aca cag act tgc ttt ccc tgt ggc tac taa tcc caa 2100

2110 2120 2130 2140 2150 2160

ttg cac att cag tcc ctt gat aga ctt act cta aaa atc taa gtt cag cgg tcc acg aaa 2160
cat aac aaa gcc tgt cct aaa aca gca aga aag aaa gaa aga aag aaa gca aga aag aaa 2220
gca aga aag aaa gaa aga aaa cag aag aca aac aag gtc ttt ccc cat tcc cta aca tac 2280
agg aat gga aat tat taa gtc tac gtg ata gcc aat gca tct gtt tct tca gta tgc cca 2340
caa ggg tgc tgc cgg agc cat tgc tca ggg ctg gag tat tta ctg ggc atg ctt gac ccc 2400

2410 2420 2430 2440 2450 2460

agc atg gag ggt gag aag tgc tcc tgg gaa ctc tga tcc act gct gtg gtg gag agc aaa 2460
cac ctg gcc tca ttt ata ctt gtt gtc tgt ata atg cat ata aat ggg gga taa tca tta 2520
cta aac tgt tta gct gag cct cat gtc agt caa tca caa agc aga gta att acc aca cag 2580
act ggg aag ctc agt gaa gat tgt tag cgg ttg gtc tga cag tct tgc tgt gtg cta tag 2640
tgt tag acc caa cgg agg cag tat tta toa gga ggg cag ggt tcc atg ttt ccc gtg tta 2700

FIG.27C

2710 2720 2730 2740 2750 2760

aag agc aag aga tga tgt ttg tca gta ggc atg cag ctc atg gtg aaa aga aag tcc aga 2760
ctt aaa gat gtg aag tga ttt gtg ctt tgc ccc acc ctg aca gtc tct ctc tgt gtg cst 2820
tca GCT GTG GTG GTG TTT GCT TTC ATC CTC TGC TGG CTG CCC TTC CAC GTG GGA AGA TAC 2880
CTC TTT TCC AAG TCC TTC GAG CCT GGC TCT CTG GAG ATC GCT CAG ATC AGC CAG TAC TGC 2940
AAC CTG GTG TCC TTT GTC CTC TTC TAC CTC AGC GCT GCC ATC AAC CCC ATT CTG TAC AAC 3000

3010 3020 3030 3040 3050 3060

ATC ATG TCC AAG AAG TAC CGG GTG GCA GTG TTC AAA CTG CTA GGA TTT GAA TCC TTC TCC 3060
CAG AGA AAG CTT TCC ACT CTG AAG GAT GAG AGT TCC CGG GCC TGG ACA AAG TCG AGC ATC 3120
AAC ACA TGA 3129

FIG.27D

```
          10          20          30          40          50          60
          .           .           .           .           .           .
ATG TGG AAC GCG ACC CCC AGC GAG GAG CCG GAG CCT AAC GTC ACG TTG GAC CTG GAT TGG    60
GAC GCT TCC CCC GGC AAC GAC TCA CTG CCT GAC GAA CTG CTG CCG CTG TTC CCC GCT CCG   120
CTG CTG GCA GGC GTC ACC GCC ACC TGC GTG GCG CTC TTC GTG GTG GGC ATC TCA GGC AAC   180
CTG CTC ACT ATG CTG GTG GTG TCC CGC TTC CGC GAG CTG CGC ACC ACC ACC AAC CTC TAC   240
CTG TCC AGC ATG GCC TTC TCG GAT CTG CTC ATC TTC CTG TGC ATG CCG CTG GAC CTC GTC   300

          310         320         330         340         350         360
          .           .           .           .           .           .
CGC CTC TGG CAG TAC CGG CCC TGG AAC TTC GGC GAC CTG CTC TGC AAA CTC TTC CAG TTT   360
GTC ACC GAG AGC TGC ACC TAC GCC ACG GTC CTC ACC ATC ACC GCG CTG AGC GTC GAG CGC   420
TAC TTC GCC ATC TGC TTC CCT CTG CGG GCC AAG GTG GTG GTC ACT AAG GGC CGC GTG AAG   480
CTG GTC ATC CTT GTC ATC TGG GCC GTG GCT TTC TGC AGC GCG GGG CCC ATC TTC GTG CTG   540
GTG GGC GTG GAG CAC GAA AAC GGC ACA GAT CCC CGG GAC ACC AAC GAA TGC CGC GCC ACC   600

          610         620         630         640         650         660
          .           .           .           .           .           .
GAG TTC GCT GTG CGC TCT GGG CTG CTC ACC GTC ATG GTG TGG GTG TCC AGC GTC TTC TTC   660
TTT CTA CCG GTC TTC TGC CTC ACT GTG CTC TAC AGT CTC ATC GGG AGG AAG CTA TGG CGG   720
AGA CGC GGA GAT GCA GCG GTG GGC GCC TCG CTC CGG GAC CAG AAC CAC AAG CAG ACA GTG   780
AAG ATG CTT GCT GTG GTG GTG TTT GCT TTC ATC CTC TGC TGG CTG CCC TTC CAC GTG GGA   840
AGA TAC CTC TTT TCC AAG TCC TTC GAG CCT GGC TCT CTG GAG ATC GCT CAG ATC AGC CAG   900
```

FIG.28A

```
        910          920          930            940          950          960
TAC TGC AAC CTG GTG TCC TTT GTC CTC TTC TAC CTC AGC GCT GCC ATC AAC CCC ATT CTG   960
TAC AAC ATC ATG TCC AAG AAG TAC CGG GTG GCA GTG TTC AAA CTG CTA GGA TTT GAA TCC  1020
TTC TCC CAG AGA AAG CTT TCC ACT CTG AAG GAT GAG AGT TCC CGG GCC TGG ACA AAG TCG  1080
AGC ATC AAC ACA  1092
```

FIG.28B

```
        10         20         30         40         50
         .          .          .          .          .
MWNATPSEEP EPNVTLDLDW DASPGNDSLP DELLPLFPAP LLAGVTATCV    50
ALFVVGISGN LLTNLVVSRF RELRTTTNLY LSSMAFSDLL IFLCMPLDLV   100
RLWQYRPWNF GDLLCKLFQF VSESCTYATV LTITALSVER YFAICFPLRA   150
KVVVTKGRVK LVILVIWAVA FCSAGPIFVL VGVEHENGTD PRDTNECRAT   200
EFAVRSGLLT VMVWVSSVFF FLPVFCLTVL YSLIGRKLWR RRGDAAVGAS   250

       260        270        280        290        300
         .          .          .          .          .
LRDQNHKQTV KMLAVVVFAF ILCWLPFHVG RYLFSKSFEP GSLEIAQISQ   300
YCNLVSFVLF YLSAAINPIL YNIMSKKYRV AVFKLLGFES FSQRKLSTLK   350
DESSRAWTKS SINT  364
```

FIG.29

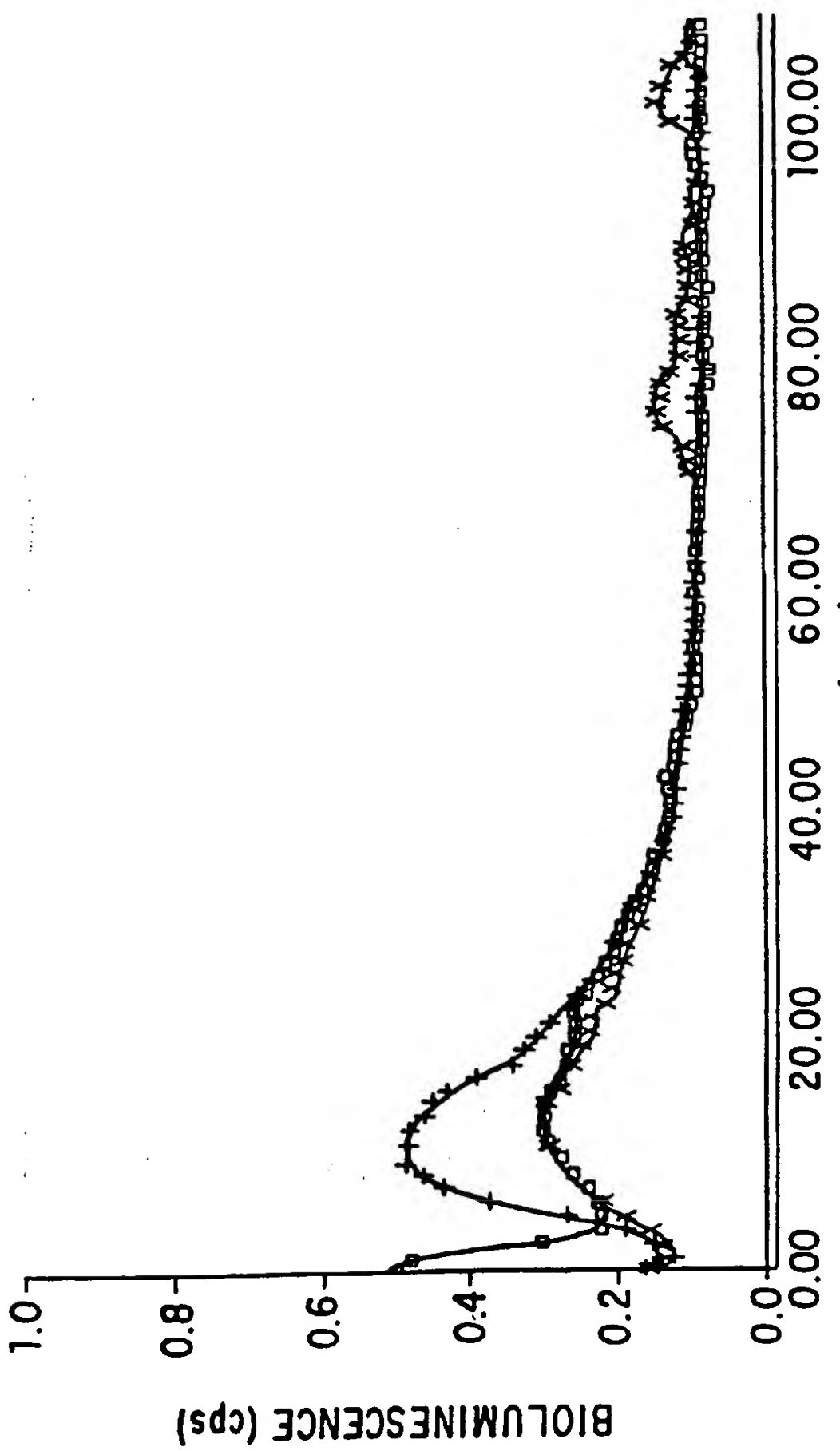
BIOLUMINESCENCE (cps)
1.0
0.8
0.6
0.4
0.2
0.0
0.00
20.00
40.00
60.00
80.00
100.00
TIME (SEC)

FIG. 30

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/19442

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : G01N 33/566; C12N 15/87, 15/63; C07K 14/715; C12Q 1/02
US CL : 435/7.21, 29, 325, 320.1; 530/350
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.21, 325, 320.1; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A, E	US 5,591,641 A (THORNER ET AL.) 07 January 1997, see column 4 line 5-63, column 19, line 10-25.	1, 3-14
A	SETHUMADHAVAN et al. Demonstration and characterization of the specific binding of growth hormone-releasing peptide to rat anterior pituitary and hypothalamic membranes. Biochem. Biophys. Res. Comm. 15 July 1991, Vol. 178, pages 31-37. See entire document.	1, 3-14
A, P	HOWARD et al. A receptor in pituitary and hypothalamus that functions in growth hormone release. Science. 16 August 1996, Vol 273, pages 974-977.	1, 3-14

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 13 FEBRUARY 1997	Date of mailing of the international search report 04 MAR 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer MUKUL RANJAN Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/19442

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 4,410,513 A (MOMANY ET AL.) 18 October 1983, see entire document.	1, 3-14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/19442

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1, 3-14

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/19442

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, MEDLINE, BIOSIS, CAPUS, JICST-EPLUS, WPIDS
Search Terms : Growth Hormone Seretagogue Receptor, Growth Hormone releasing peptide, HRP, GHSR. Assay , G-protein receptor,

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups ofinventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s)1, 3-14, drawn to an assay to identify GHSR or GHSRR.
Group II, claim(s) 2, 15-18, drawn to an assay to identify growth hormone secretagogues.
Group III, claim(s) 19-22, drawn to a binding assay for ligand.
Group IV, claim(s) 23, drawn to ligands identified in assay of group II.
Group V, claim(s) 24, drawn to a binding assay for growth hormone secretagogue receptors.

The inventions listed as Groups I-V do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Groups I, II, III and V are to processes that either use materially different process steps or different products which are their special technical features. Pursuant to 37 CFR 1- 475(d), the ISA/US considers that unity of invention of invention does not exist between dissimilar methods which do not correspond to the main invention of the first group.

The ligands of Group IV is structurally and functionally different from, and does not share the special technical feature of the methods of groups I, III and V which are the process steps used by the methods.

Group IV does not share the special technical features of either of groups II or III because neither II nor II distinguish prior
art products meeting functional requirements of IV.